

**IL-1 delta DNA AND POLYPEPTIDES****CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a continuation-in-part of USSN 09/612,921, filed July 10, 2000. This application also claims the benefit of U.S. Provisional Application Serial No. 60/071,074, filed January 9, 1998, and U.S. Provisional Application Serial No. 60/087,393, filed June 1, 1998. The disclosures of these applications are hereby incorporated by reference.

**BACKGROUND OF THE INVENTION****Field of the Invention**

The invention is directed to purified and isolated novel IL-1 delta polypeptides and fragments thereof, the nucleic acids encoding such polypeptides, processes for production of recombinant forms of such polypeptides, antibodies generated against these polypeptides, peptides derived from these polypeptides, and uses thereof.

**Description of Related Art****1. IL-1 and IL-1R**

Interleukin-1 (IL-1) is a member of a large group of cytokines whose primary function is to mediate immune and inflammatory responses. Members of the IL-1 ligand family include IL-1 alpha (IL-1 $\alpha$ ), IL-1 beta (IL-1 $\beta$ ), IL-1 receptor antagonist (IL-1ra), and IL-18 (previously known as IGIF and sometimes IL-1 gamma), IL-1 epsilon (IL-1 $\epsilon$ ), and IL-1 zeta (IL-1 $\zeta$ ). IL-1 that is secreted by macrophages is actually a mixture of mostly IL-1 $\beta$  and some IL-1 $\alpha$  (Abbas et al., 1994). IL-1 $\alpha$  and IL-1 $\beta$ , which are first produced as 33 kD precursors that lack a signal sequence, are further processed by proteolytic cleavage to produce secreted active forms, each about 17 kD. Additionally, the 33 kD precursor of IL-1 $\alpha$  is also active. Both forms of IL-1 are the products of two different genes located on chromosome 2. Although the two forms are less than 30 percent homologous to each other, they both bind to the same receptors and have similar activities.

IL-1ra, a biologically inactive form of IL-1, is structurally homologous to IL-1 and binds to the same receptors. Additionally, IL-1ra is produced with a signal sequence which allows for efficient secretion into the extracellular region where it competitively competes with IL-1 (Abbas et al., 1994).

The IL-1 type I receptor mediates the biological effects of IL-1. Activities attributed to IL-1 $\alpha$  and IL-1 $\beta$  include induction of inflammatory cytokines and other inflammatory responses including prostaglandins, nitric oxide, metalloproteinases, adhesion molecules, acute phase proteins, hematopoiesis, fever, bone resorption, and Th2 cell growth and differentiation.

IL-1 has been implicated in chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease. There is increasing evidence that IL-1 plays a role in osteoporosis. All of these activities are initiated by the signaling function of the cytoplasmic portion of the type I IL-1R.

IL-18 is a homolog of IL-1 $\alpha$  and IL-1 $\beta$ , and binds to and signals through a receptor comprised of the IL-1 family members IL-1 receptor related protein 1, IL-1Rrp1 (See Parnet et al., *J. Biol. Chem.* 271:3967, 1996, and Torigoe et al., *J. Biol. Chem.* 272:25737, 1997), and AcPL (See U. S. Provisional Patent Application 60/072,301 of John E. SIMS and Teresa L. BORN for ACPL DNA and Polypeptides filed January 23, 1998, and U.S. Provisional Application No. 60/078,835, of John E. SIMS and Teresa L. BORN for ACPL DNA and Polypeptides filed March 20, 1998, both of which are hereby incorporated by reference). IL-18 acts as a stimulator of Th1 cell growth and differentiation, and is a potent inducer of  $\gamma$ -interferon production from Th1 cells. It enhances NK cell killing activity. It has been implicated in septic shock, liver destruction, inflammatory bowel disease, and diabetes.

IL-1 $\alpha$  and IL-1 $\beta$  bind to two IL-1 receptors, which are members of the Ig superfamily. IL-1 receptors include the 80 kDa type I receptor (IL-1RI), and a 68 kDa type II receptor (IL-1RII). IL-1 $\alpha$  and IL-1 $\beta$  can also bind to a soluble proteolytic fragment of IL-1RII (sIL-1RII) (Colotta et al., 1993).

IL-1 receptors are members of the large Ig superfamily of cytokine receptors, many of which mediate the response of immune system cells, in particular lymphocytes. In recent years, members of the ligands that bind to these receptors have been discovered at an accelerated pace. The increase in the number of known IL-1 ligands has been largely due to the advent of gene cloning and sequencing techniques. Amino acid sequences deduced from nucleotide sequences are considered to represent IL-1 ligands if they share homology with other known IL-1 ligands.

The major source of IL-1 is the activated macrophage or mononuclear phagocyte. Other cells that produce IL-1 include epithelial and endothelial cells (Abbas et al., 1994). IL-1 secretion from macrophages occurs after the macrophage encounters and ingests gram-negative bacteria. Such bacteria contain lipopolysaccharide (LPS) molecules, also known as endotoxin, in the bacterial cell wall. LPS molecules are the active components that stimulate macrophages to produce tumor necrosis factor (TNF) and IL-1. In this case, IL-1 is produced in response to LPS and TNF production. At low concentrations, LPS stimulates macrophages and activates B-cells and other host responses needed to eliminate the bacterial infection; however, at high concentrations, LPS can cause severe tissue damage, shock, and even death.

The biological functions of IL-1 include activating vascular endothelial cells and lymphocytes, local tissue destruction, and fever (Janeway et al., 1996). At low levels, IL-1 stimulates macrophages and vascular endothelial cells to produce IL-6, upregulates molecules on the surface of vascular endothelial cells to increase leukocyte adhesion, and indirectly activates inflammatory leukocytes by stimulating mononuclear phagocytes and other cells to produce certain chemokines that activate inflammatory leukocytes. Additionally, IL-1 is involved in other inflammatory responses such as induction of prostaglandins, nitric oxide synthetase, and metalloproteinases. These IL-1 functions are crucial during low level microbial infections. However, if the microbial infection escalates, IL-1 acts systemically by inducing fever, stimulating mononuclear phagocytes to produce IL-1 and IL-6, increasing the production of serum proteins from hepatocytes, and activating the coagulation system. Additionally, IL-1 does not cause hemorrhagic necrosis of tumors or suppress bone marrow stem cell division, and IL-1 is lethal to humans at high concentrations.

IL-1 mediated cellular signaling often involves a molecular activation cascade, during which a receptor propagates a ligand-receptor mediated signal by specifically activating intracellular kinases which phosphorylate target substrates. IL-1 mediated cellular signaling may result in the activation of the transcription factors NFkappaB and AP1 (Stylianou et al., *Int. J. Biochem Cell Biol.* 30: 1075-1079, 1998), the protein kinases Jun N-terminal kinase and p38 map kinase (O'Neil et al., *J. Leukoc. Biol.* 63:650-657, 1998), the enzymes COX-2 leading to prostaglandin production (Crofford, *J. Rheumatol.* 24 Suppl. 49:15-19, 1997) and iNOS leading to nitric oxide production (Alexander, *Nutrition* 14: 376-90, 1998) and inflammation in general.

Given the important function of IL-1 and IL-1R, there is a need in the art for additional members of the IL-1 ligand family. Despite the growing body of knowledge, there is still a need in the art for the identity and function of proteins involved in cellular and immune responses.

### **SUMMARY OF THE INVENTION**

The invention aids in fulfilling these various needs in the art by providing isolated IL-1 delta nucleic acids and polypeptides encoded by these nucleic acids. Particular embodiments of the invention are directed to an isolated IL-1 delta nucleic acid molecule comprising the murine DNA sequence of SEQ ID NO:1 and the human DNA sequence of SEQ ID NO:3 and an isolated IL-1 delta nucleic acid molecule encoding the corresponding amino acid sequences of SEQ ID NO:2 or SEQ ID NO:4, as well as nucleic acid molecules complementary to these sequences. Both single-stranded and double-stranded RNA and DNA nucleic acid molecules are encompassed by the invention, as well as nucleic acid molecules that hybridize to a denatured, double-stranded DNA comprising all or a portion of SEQ ID NO:1 or SEQ ID NO:3. Also encompassed are isolated nucleic acid molecules that are derived by *in vitro* mutagenesis of nucleic acid molecules comprising sequences of SEQ ID NO:1 or SEQ ID NO:3, that are degenerate from nucleic acid molecules comprising sequences of SEQ ID NO:1 or SEQ ID NO:3, and that are allelic variants of DNA of the invention. The invention also encompasses recombinant vectors that direct the expression of these nucleic acid molecules and host cells stably or transiently transformed or transfected with these vectors.

In addition, the invention encompasses methods of using the nucleic acids noted above to identify nucleic acids encoding proteins having IL-1 delta activity; to identify human chromosome 2; to map genes on human chromosome 2, to identify genes associated with certain diseases, syndromes, or other human conditions associated with human chromosome 2; and to study cell signal transduction and the IL-1 delta system.

The invention also encompasses the use of sense or antisense oligonucleotides from the nucleic acid of SEQ ID NO:1 or SEQ ID NO:3 to inhibit the expression of the polynucleotide encoded by the IL-1 delta gene.

The invention also encompasses isolated polypeptides and fragments thereof encoded by these nucleic acid molecules including soluble polypeptide portions of SEQ ID NO:2 or SEQ ID NO:4. The invention further encompasses methods for the production of these polypeptides, including culturing a host cell under conditions promoting expression and recovering the polypeptide from the culture medium.

Especially, the expression of these polypeptides in bacteria, yeast, plant, insect, and animal cells is encompassed by the invention.

In general, the polypeptides of the invention can be used to study cellular processes such as immune regulation, cell proliferation, cell death, cell migration, cell-to-cell interaction, and inflammatory responses. In addition, these polypeptides can be used to identify proteins associated with IL-1 delta ligands and IL-1 delta receptors.

In addition, the invention includes assays utilizing these polypeptides to screen for potential inhibitors of activity associated with polypeptide counter-structure molecules, and methods of using these polypeptides as therapeutic agents for the treatment of diseases mediated by IL-1 delta polypeptide counter-structure molecules. Further, methods of using these polypeptides in the design of inhibitors thereof are also an aspect of the invention.

Further encompassed by this invention is the use of the IL-1 delta nucleic acid sequences, predicted amino acid sequences of the polypeptide or fragments thereof, or a combination of the predicted amino acid sequences of the polypeptide and fragments thereof for use in searching an electronic database to aid in the identification of sample nucleic acids and/or proteins.

Isolated polyclonal or monoclonal antibodies that bind to these polypeptides are also encompassed by the invention, in addition the use of these antibodies to aid in purifying the IL-1 delta polypeptide.

The invention also encompasses isolated polypeptides encoded by these nucleic acid molecules, including isolated polypeptides having a molecular weight of approximately 17 kD as determined by SDS-PAGE and isolated polypeptides in non-glycosylated form.

#### **DETAILED DESCRIPTION OF THE INVENTION**

A cDNA encoding mouse IL-1 delta polypeptide has been isolated and is disclosed in SEQ ID NO:1.

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25 ATGATGGTTCTGAGTGGGGCACTATGCTTCCGAATGAAGGATTCAGCCTT
   GAAGGTACTGTATCTGCACAATAACCAGCTGCTGGCTGGAGGACTGCACG
   CAGAGAAGGTCATTAAAGGTGAGGAGATCAGTGTGTCCCAAATCGGGCA
   CTGGATGCCAGCTGTCTCCCTGTCACTCTGGCGTTCAAGGAGGAAGCCA
   GTGCCTATCTTGTGGGACAGAGAAAGGCCAATCTTGAACCTTGAGCCAG
30 TGAACATCATGGAGCTCTACCTCGGGGCCAAGGAATCAAAGAGCTTCACC
   TTCTACCGGCGGATATGGGTCTTACCTCCAGCTTCGAATCCGCTGCCTA
   CCCAGGCTGGTTCTCTGCACCTCACCGGAAGCTGACCAGCCTGTGAGGC
   TCACTCAGATCCCTGAGGACCCGCTGGGATGCTCCCATCACAGACTTC
   TACTTTCAGCAGTGTGAC (SEQ ID NO:1)

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A cDNA encoding human IL-1 delta polypeptide has been isolated and is disclosed in SEQ ID NO:3.

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ATGGTCCTGA GTGGGCGCT GTGCTTCCGA ATGAAGGACT CGGCATTGAA
GGTGCTTTAT CTGCATAATA ACCAGCTTCT AGCTGGAGGG CTGCATGCAG
GGAAGGTCAT TAAAGGTGAA GAGATCAGCG TGGTCCCCAA TCGGTGGCTG
40 GATGCCAGCC TGTCCCCGT CATCTGGGT GTCCAGGGTG GAAGCCAGTG

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CCTGTCATGT GGGGTGGGGC AGGAGCCGAC TCTAACACTA GAGCCAGTGA  
 ACATCATGGA GCTCTATCTT GGTGCCAAGG AATCCAAGAG CTTCACTTC  
 TACCGGCGGG ACATGGGGCT CACCTCCAGC TTCGAGTCGG CTCGCTACCC  
 GGGCTGGTTC CTGTGCACGG TGCCTGAAGC CGATCAGCCT GTCAGACTCA  
 5 CCCAGCTTCC CGAGAATGGT GGCTGGAATG CCCCCATCAC AGACTTCTAC  
 TTCCAGCAGT GTGACTAG (SEQ ID NO:3)

This discovery of the cDNAs encoding IL-1 delta polypeptides enables construction of expression vectors comprising nucleic acid sequences encoding IL-1 delta polypeptides; host cells transfected or transformed with the expression vectors; biologically active IL-1 delta polypeptides and IL-1 delta  
 10 molecular weight markers as isolated and purified proteins; and antibodies immunoreactive with IL-1 delta polypeptides. In addition, understanding of the mechanism by which IL-1 delta functions in IL-1 signaling enables the design of assays to detect inhibitors of IL-1 activity.

Mouse IL-1 delta DNA was originally seen as a partial EST clone in the public databases (mouse EST W08205). The DNA sequence of the entire IMAGE clone was determined. The sequence of EST  
 15 clones W08205 and W20594 overlaps with nucleotides of mouse IL-1 delta DNA (SEQ ID NO:1). No other homologous EST sequences have been identified.

Murine mRNA expression has been demonstrated in spleen cells stimulated with CD40L. The expression is further augmented by stimulation with both lipopolysaccharide (LPS) and CD40L. mRNA expression has been demonstrated in a mouse macrophage line (RAW) stimulated with LPS and in both the  
 20 mouse placenta and yolk sac of a 14 day embryo. The cDNA has been detected in a kidney cDNA library.

Human IL-1 delta RNA expression can be detected in lymph node, thymus, tonsil, brain, placenta, lung, skeletal muscle, prostate, and testis.

A preferred polypeptide encoded by SEQ ID NO:1 is set forth below:

MMVLGALCFR MKDSALKVLYLHNNQLLAGLHAEKVIKGEIISVVPNRA  
 25 LDASLSPVILGVQGSQCLSCGTEKGPILKLEFPVNMIMELYLGAKESKSTP  
 FYRRDMGLTSSFESAAYPGWFLCTSP EADQPVRLTQIPEDPAWDAPITDF  
 YFQQCD (SEQ ID NO:2)

A preferred polypeptide encoded by SEQ ID NO:3 is set forth below:

MVLGALCFR MKDSALKVLY LHHNQLLAGG LHAGKVIKGE EISVVPNRWL  
 30 DASLSPVILG VQGSQCLSC GVGQEP TLT L EPVNMIMELYL GAKESKSTP  
 YRRDMGLTSS FESAAYPGWF LCTVPEADQP VRLTQLPENG GWNAPITDFY  
 FQQCD (SEQ ID NO:4)

Mouse IL-1 delta polypeptide is homologous to the mature portion of the IL-1 family members (IL-1 $\alpha$ , IL-1 $\beta$ , IL-1 $\alpha$ , and IL-18). The mouse IL-1 delta amino acid sequence was compared to the mature  
 35 forms of the other mouse IL-1 family members using the UWGCG computer program "gap." Mouse IL-1 delta polypeptide exhibited 23% identity with IL-1 $\alpha$ , 30% identity with IL-1 $\beta$ , 48% identity with IL-1 $\alpha$ , and 18% identity with IL-18.

The human IL-1 delta amino acid sequence was compared to the mature forms of the other human IL-1 family members. Human IL-1 delta polypeptide exhibited little identity with IL-1 $\alpha$ , 29% identity with  
 40 IL-1 $\beta$ , 50% identity with IL-1 $\alpha$ , little identity with IL-18, 31% identity with IL-1 epsilon, and 34% with

IL-1 zeta, as determined by the UWGCG program GAP, using a gap creation penalty of 12 and a gap extension penalty of 4.

Unlike IL-1 $\alpha$ , IL-1 $\beta$ , and IL-18, the IL-1 delta polypeptide does not contain a "pro" region (an N-terminal segment, which is removed by proteolytic processing). Unlike IL-1 $\alpha$ , IL-1 delta polypeptide does not contain a signal peptide.

A secreted version of mouse IL-1 delta was generated by fusing the IL-1 $\alpha$  signal peptide to the coding region. Inefficient protein production was found in the COS and CV1/EBNA mammalian systems and in bacterial systems.

Human IL-1 delta is secreted with moderate efficiency from transfected COS or CV-1/EBNA cells, even in the absence of an exogenous signal peptide. Sequencing of the N-terminus indicates that no cleavage has occurred. Therefore, native IL-1 delta can be secreted from the cell even in the absence of a signal peptide or a "pro" region.

A soluble version of IL-1 delta may act as an antagonist of other, active cytokines, in the same way that IL-1 $\alpha$  is an antagonist of the actions of IL-1 alpha and IL-1 beta.

The discovery of the nucleic acids of the invention enables the construction of expression vectors comprising nucleic acid sequences encoding polypeptides; host cells transfected or transformed with the expression vectors; isolated and purified biologically active polypeptides and fragments thereof; the use of the nucleic acids or oligonucleotides thereof as probes to identify nucleic acid encoding proteins having IL-1 delta activity. The invention also provides for the use of the nucleic acids or oligonucleotides thereof to identify human chromosome 2, to map genes on human chromosome 2, and to identify genes associated with certain diseases, syndromes or other human conditions associated with human chromosome 2, particularly with region 2q11-12, including glaucoma, ectodermal dysplasia, insulin-dependent diabetes mellitus, wrinkly skin syndrome, T-cell leukemia/lymphoma, and tibial muscular dystrophy. In another aspect, the invention describes the use of single-stranded sense or antisense oligonucleotides from the nucleic acids to inhibit expression of polynucleotide encoded by the IL-1 delta gene. The invention also enables the use of such polypeptides and soluble fragments as molecular weight markers, as controls for peptide fragmentation, as well as kits comprising these reagents. Finally, the invention provides for the use of such polypeptides and fragments thereof to generate antibodies, and the use of antibodies to purify the IL-1 delta polypeptide.

#### NUCLEIC ACID MOLECULES

In a particular embodiment, the invention relates to certain isolated nucleotide sequences that are free from contaminating endogenous material. A "nucleotide sequence" refers to a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct. The nucleic acid molecule has been derived from DNA or RNA isolated at least once in substantially pure form and in a quantity or concentration enabling identification, manipulation, and recovery of its component nucleotide sequences by standard biochemical methods (such as those outlined in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)). Such sequences are preferably provided and/or constructed in the form of an open reading frame uninterrupted by

internal non-translated sequences, or introns, that are typically present in eukaryotic genes. Sequences of non-translated DNA can be present 5' or 3' from an open reading frame, where the same do not interfere with manipulation or expression of the coding region.

Nucleic acid molecules of the invention include DNA in both single-stranded and double-stranded form, as well as the RNA complement thereof. DNA includes, for example, cDNA, genomic DNA, chemically synthesized DNA, DNA amplified by PCR, and combinations thereof. Genomic DNA may be isolated by conventional techniques, *e.g.*, using the cDNA of SEQ ID NO:1 or SEQ ID NO:3, or a suitable fragment thereof, as a probe.

The DNA molecules of the invention include full length genes as well as polynucleotides and fragments thereof. The nucleic acids of the invention are derived from either murine or human sources, but the invention includes those derived from other sources as well.

#### Preferred Sequences

Particularly preferred nucleotide sequences of the invention are the murine sequence set forth in SEQ ID NO:1 and the human sequence set forth in SEQ ID NO:3. The sequences of amino acids encoded by the DNA of SEQ ID NO:1 and SEQ ID NO:3 are shown in SEQ ID NO:2 and SEQ ID NO:4, respectively. These sequences identify IL-1 delta polynucleotides as members of the IL-1 receptor family.

#### Additional Sequences

Due to the known degeneracy of the genetic code, wherein more than one codon can encode the same amino acid, a DNA sequence can vary from that shown in SEQ ID NO:1 or SEQ ID NO:3, and still encode a polypeptide having the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4. Such variant DNA sequences can result from silent mutations (*e.g.*, occurring during PCR amplification), or can be the product of deliberate mutagenesis of a native sequence.

The invention thus provides isolated DNA sequences encoding polypeptides of the invention, selected from: (a) DNA comprising the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3; (b) DNA encoding the polypeptides of SEQ ID NO:2 or SEQ ID NO:4; (c) DNA capable of hybridization to a DNA of (a) or (b) under conditions of moderate stringency and which encodes polypeptides of the invention; (d) DNA capable of hybridization to a DNA of (a) or (b) under conditions of high stringency and which encodes polypeptides of the invention, and (e) DNA which is degenerate as a result of the genetic code to a DNA defined in (a), (b), (c), or (d) and which encode polypeptides of the invention. Of course, polypeptides encoded by such DNA sequences are encompassed by the invention.

The invention thus provides equivalent isolated DNA sequences encoding biologically active IL-1 delta polypeptides selected from: (a) DNA derived from the coding region of a native mammalian IL-1 delta gene; (b) DNA selected from the group consisting of SEQ ID NO:1 or SEQ ID NO:3, (c) DNA capable of hybridization to a DNA of (a) or (b) under conditions of moderate stringency and which encodes biologically active IL-1 delta polypeptides; and (d) DNA that is degenerate as a result of the genetic code to a DNA defined in (a), (b) or (c), and which encodes biologically active IL-1 delta polypeptides. IL-1 delta polypeptides encoded by such DNA equivalent sequences are encompassed by the invention. IL-1 delta

polypeptides encoded by DNA derived from other mammalian species, wherein the DNA will hybridize to the complement of the DNA of SEQ ID NO:1 or SEQ ID NO:3, are also encompassed.

As used herein, conditions of moderate stringency can be readily determined by those having ordinary skill in the art based on, for example, the length of the DNA. The basic conditions are set forth by Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989), and include use of a prewashing solution for the nitrocellulose filters 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of about 50% formamide, 6X SSC at about 42°C (or other similar hybridization solution, such as Stark's solution, in about 50% formamide at about 42°C), and washing conditions of about 60°C, 0.5X SSC, 0.1% SDS. Conditions of high stringency can also be readily determined by the skilled artisan based on, for example, the length of the DNA. Generally, such conditions are defined as hybridization conditions as above, and with washing at approximately 68°C, 0.2X SSC, 0.1% SDS. The skilled artisan will recognize that the temperature and wash solution salt concentration can be adjusted as necessary according to factors such as the length of the probe.

Also included as an embodiment of the invention is DNA encoding polypeptide fragments and polypeptides comprising inactivated N-glycosylation site(s), inactivated protease processing site(s), or conservative amino acid substitution(s), as described below.

In another embodiment, the nucleic acid molecules of the invention also comprise nucleotide sequences that are at least 80% identical to a native sequence. Also contemplated are embodiments in which a nucleic acid molecule comprises a sequence that is at least 90% identical, at least 95% identical, at least 98% identical, at least 99% identical, or at least 99.9% identical to a native sequence.

The percent identity may be determined by visual inspection and mathematical calculation. Alternatively, the percent identity of two nucleic acid sequences can be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Other programs used by one skilled in the art of sequence comparison may also be used.

The invention also provides isolated nucleic acids useful in the production of polypeptides. Such polypeptides may be prepared by any of a number of conventional techniques. A DNA sequence encoding a IL-1 delta polypeptide, or desired fragment thereof, may be subcloned into an expression vector for production of the polypeptide or fragment. The DNA sequence advantageously is fused to a sequence encoding a suitable leader or signal peptide. Alternatively, the desired fragment may be chemically synthesized using known techniques. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. If necessary, oligonucleotides that reconstruct the 5' or 3' terminus to a desired point may be ligated to a DNA fragment generated by restriction enzyme digestion. Such oligonucleotides may additionally contain a



restriction endonuclease cleavage site upstream of the desired coding sequence, and position an initiation codon (ATG) at the N-terminus of the coding sequence.

The well-known polymerase chain reaction (PCR) procedure also may be employed to isolate and amplify a DNA sequence encoding a desired protein fragment. Oligonucleotides that define the desired termini of the DNA fragment are employed as 5' and 3' primers. The oligonucleotides may additionally contain recognition sites for restriction endonucleases, to facilitate insertion of the amplified DNA fragment into an expression vector. PCR techniques are described in Saiki et al., *Science* 239:487 (1988); *Recombinant DNA Methodology*, Wu et al., eds., Academic Press, Inc., San Diego (1989), pp. 189-196; and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc. (1990).

#### POLYPEPTIDES AND FRAGMENTS THEREOF

The invention encompasses polypeptides and fragments thereof in various forms, including those that are naturally occurring or produced through various techniques such as procedures involving recombinant DNA technology. For example, DNAs encoding IL-1 delta polypeptides can be derived from SEQ ID NO:1 or SEQ ID NO:3 by *in vitro* mutagenesis, which includes site-directed mutagenesis, random mutagenesis, and *in vitro* nucleic acid synthesis. Such forms include, but are not limited to, derivatives, variants, and oligomers, as well as fusion proteins or fragments thereof.

#### Polypeptides and Fragments Thereof

The polypeptides of the invention include full length proteins encoded by the nucleic acid sequences set forth above. Particularly preferred polypeptides comprise the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4.

The polypeptides of the invention may be membrane bound or they may be secreted and thus soluble. Soluble polypeptides are capable of being secreted from the cells in which they are expressed. In general, soluble polypeptides may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells which express the desired polypeptide from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired polypeptide. The presence of polypeptide in the medium indicates that the polypeptide was secreted from the cells and thus is a soluble form of the protein.

In general, the use of soluble forms is advantageous for certain applications. Purification of the polypeptides from recombinant host cells is facilitated, since the soluble polypeptides are secreted from the cells. Further, soluble polypeptides are generally more suitable for intravenous administration.

The invention also provides polypeptides and fragments of the IL-1 delta that retain a desired biological activity. Particular embodiments are directed to polypeptide fragments that retain the ability to bind IL-1 delta counter-structures, such as IL-1 family members. Such a fragment may be a soluble polypeptide, as described above. In another embodiment, the polypeptides and fragments advantageously include regions that are conserved in the IL-1 delta family as described above.

Also provided herein are polypeptide fragments of varying lengths. In one embodiment, a preferred IL-1 delta polypeptide fragment comprises at least 6 contiguous amino acids of an amino acid

sequence. In other embodiments, a preferred IL-1 delta polypeptide fragment comprises at least 10, at least 20, at least 30, up to at least 100 contiguous amino acids of the amino acid sequences of SEQ ID NO:2 and/or SEQ ID NO:4. These polypeptides can be produced in soluble form. In addition, fragments find use in studies of signal transduction, and in regulating cellular processes associated with transduction of biological signals. Polypeptide fragments also may be employed as immunogens, in generating antibodies.

#### Variants

Naturally occurring variants as well as derived variants of the polypeptides and fragments are provided herein.

An "IL-1 delta variant" as referred to herein means a polypeptide substantially homologous to native IL-1 delta polypeptide, but which has an amino acid sequence different from that of native IL-1 delta polypeptide (human, murine or other mammalian species) because of one or more deletions, insertions, or substitutions. The variant has an amino acid sequence that preferably is at least 80% identical to a native IL-1 delta polypeptide amino acid sequence, most preferably at least 90% identical. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Variants also include embodiments in which a polypeptide or fragment comprises an amino acid sequence that is at least 90% identical, at least 95% identical, at least 98% identical, at least 99% identical, or at least 99.9% identical to the preferred polypeptide or fragment thereof. Percent identity may be determined as above. Alternatively, the percent identity of two protein sequences can be determined by comparing sequence information using the GAP computer program, based on the algorithm of Needleman and Wunsch (*J. Mol. Bio.* 48:443, 1970) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a scoring matrix, *blosum62*, as described by Henikoff and Henikoff (*Proc. Natl. Acad. Sci. USA* 89:10915, 1992); (2) a gap weight of 12; (3) a gap length weight of 4; and (4) no penalty for end gaps. Other programs used by one skilled in the art of sequence comparison may also be used.

The variants of the invention include, for example, those that result from alternate mRNA splicing events or from proteolytic cleavage. Alternate splicing of mRNA may, for example, yield a truncated but biologically active protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the protein (generally from

1-5 terminal amino acids). Proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

As stated above, the invention provides isolated and purified, or homogeneous, IL-1 delta polypeptides, both recombinant and non-recombinant. Variants and derivatives of native IL-1 delta proteins that retain the desired biological activity can be obtained by mutations of nucleotide sequences coding for native IL-1 delta polypeptides. Alterations of the native amino acid sequence can be accomplished by any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene, wherein predetermined codons can be altered by substitution, deletion, or insertion. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462, all of which are incorporated by reference.

IL-1 delta polypeptides can be modified to create IL-1 delta derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, polyethylene glycol (PEG) groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of IL-1 delta polypeptides can be prepared by linking the chemical moieties to functional groups on IL-1 delta amino acid side chains or at the N-terminus or C-terminus of a IL-1 delta polypeptide or the extracellular domain thereof. Other derivatives of IL-1 delta polypeptides within the scope of this invention include covalent or aggregative conjugates of IL-1 delta polypeptides or their fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate can comprise a signal or leader polypeptide sequence (e.g. the  $\alpha$ -factor leader of *Saccharomyces*) at the N-terminus of a IL-1 delta polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

Conjugates comprising diagnostic (detectable) or therapeutic agents attached thereto are contemplated herein, as discussed in more detail below.

Other derivatives include covalent or aggregative conjugates of the polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with oligomers. Further, fusion proteins can comprise peptides added to facilitate purification and identification. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *BioTechnology* 6:1204, 1988. One such peptide is the FLAG<sup>®</sup> peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma

designated 4E11 produces a monoclonal antibody that binds the FLAG<sup>®</sup> peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the FLAG<sup>®</sup> peptide are available from Eastman Kodak Co.,

5 Scientific Imaging Systems Division, New Haven, Connecticut.

Among the variant polypeptides provided herein are variants of native polypeptides that retain the native biological activity or the substantial equivalent thereof. One example is a variant that binds with essentially the same binding affinity as does the native form. Binding affinity can be measured by conventional procedures, *e.g.*, as described in U.S. Patent No. 5,512,457 and as set forth below.

10 Variants include polypeptides that are substantially homologous to the native form, but which have an amino acid sequence different from that of the native form because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native sequence.

15 A given amino acid may be replaced, for example, by a residue having similar physicochemical characteristics. Examples of such conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another; substitutions of one polar residue for another, such as between Lys and Arg, Glu and Asp, or Gln and Asn; or substitutions of one aromatic residue for another, such as Phe, Trp, or Tyr for one another. Other conservative substitutions, *e.g.*, involving substitutions of entire regions having similar hydrophobicity characteristics, are well known.

20 Similarly, the DNAs of the invention include variants that differ from a native DNA sequence because of one or more deletions, insertions or substitutions, but that encode a biologically active polypeptide.

The invention further includes polypeptides of the invention with or without associated native-pattern glycosylation. Polypeptides expressed in yeast or mammalian expression systems (*e.g.*, COS-1 or 25 COS-7 cells) can be similar to or significantly different from a native polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of polypeptides of the invention in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules. Further, a given preparation may include multiple differentially glycosylated species of the protein. Glycosyl groups can be removed through conventional methods, in particular those utilizing glycopeptidase. In general, 30 glycosylated polypeptides of the invention can be incubated with a molar excess of glycopeptidase (Boehringer Mannheim).

Correspondingly, similar DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences are encompassed by the invention. For example, N-glycosylation sites in the polypeptide extracellular domain can be modified 35 to preclude glycosylation, allowing expression of a reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate substitutions, additions, or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that

Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Alternatively, the Ser or Thr can be replaced with another amino acid, such as Ala. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

In another example of variants, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon folding or renaturation.

Other variants are prepared by modification of adjacent dibasic amino acid residues, to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

#### Oligomers

Encompassed by the invention are oligomers or fusion proteins that contain IL-1 delta polypeptides. Such oligomers may be in the form of covalently-linked or non-covalently-linked multimers, including dimers, trimers, or higher oligomers. As noted above, preferred polypeptides are soluble and thus these oligomers may comprise soluble polypeptides. In one aspect of the invention, the oligomers maintain the binding ability of the polypeptide components and provide therefor, bivalent, trivalent, etc., binding sites.

One embodiment of the invention is directed to oligomers comprising multiple polypeptides joined via covalent or non-covalent interactions between peptide moieties fused to the polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of the polypeptides attached thereto, as described in more detail below.

#### Immunoglobulin-based Oligomers

As one alternative, an oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (PNAS USA 88:10535, 1991); Byrn et al. (Nature 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a dimer comprising two fusion proteins created by fusing a polypeptide of the invention to an Fc polypeptide derived from an antibody. A gene fusion encoding the polypeptide/Fc fusion protein is inserted into an appropriate expression vector. Polypeptide/Fc fusion proteins are expressed in host cells transformed with the recombinant expression

vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent molecules.

The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides made up of the Fc region of an antibody comprising any or all of the CH domains of the Fc region. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included. Preferred polypeptides comprise an Fc polypeptide derived from a human IgG1 antibody.

One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994) incorporated herein by reference. The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

In other embodiments, the polypeptides of the invention may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form an oligomer with as many as four IL-1 delta extracellular regions.

#### Peptide-linker Based Oligomers

Alternatively, the oligomer is a fusion protein comprising multiple polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences of the invention, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between the sequences. In particular embodiments, a fusion protein comprises from two to four soluble IL-1 delta polypeptides, separated by peptide linkers.

#### Leucine-Zippers

Another method for preparing the oligomers of the invention involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize.

The zipper domain (also referred to herein as an oligomerizing, or oligomer-forming, domain) comprises a repetitive heptad repeat, often with four or five leucine residues interspersed with other amino acids. Examples of zipper domains are those found in the yeast transcription factor GCN4 and a heat-stable DNA-binding protein found in rat liver (C/EBP; Landschulz et al., *Science* 243:1681, 1989). Two nuclear

transforming proteins, *fos* and *jun*, also exhibit zipper domains, as does the gene product of the murine proto-oncogene, *c-myc* (Landschulz et al., *Science* 240:1759, 1988). The products of the nuclear oncogenes *fos* and *jun* comprise zipper domains that preferentially form heterodimer (O'Shea et al., *Science* 245:646, 1989, Turner and Tjian, *Science* 243:1689, 1989). The zipper domain is necessary for biological activity (DNA binding) in these proteins.

The fusogenic proteins of several different viruses, including paramyxovirus, coronavirus, measles virus and many retroviruses, also possess zipper domains (Buckland and Wild, *Nature* 338:547, 1989; Britton, *Nature* 353:394, 1991; Delwart and Mosialos, *AIDS Research and Human Retroviruses* 6:703, 1990). The zipper domains in these fusogenic viral proteins are near the transmembrane region of the proteins; it has been suggested that the zipper domains could contribute to the oligomeric structure of the fusogenic proteins. Oligomerization of fusogenic viral proteins is involved in fusion pore formation (Spruce et al, *Proc. Natl. Acad. Sci. U.S.A.* 88:3523, 1991). Zipper domains have also been recently reported to play a role in oligomerization of heat-shock transcription factors (Rabindran et al., *Science* 259:230, 1993).

Zipper domains fold as short, parallel coiled coils (O'Shea et al., *Science* 254:539; 1991). The general architecture of the parallel coiled coil has been well characterized, with a "knobs-into-holes" packing as proposed by Crick in 1953 (*Acta Crystallogr.* 6:689). The dimer formed by a zipper domain is stabilized by the heptad repeat, designated  $(abcdefg)_n$  according to the notation of McLachlan and Stewart (*J. Mol. Biol.* 98:293; 1975), in which residues *a* and *d* are generally hydrophobic residues, with *d* being a leucine, which line up on the same face of a helix. Oppositely-charged residues commonly occur at positions *g* and *e*. Thus, in a parallel coiled coil formed from two helical zipper domains, the "knobs" formed by the hydrophobic side chains of the first helix are packed into the "holes" formed between the side chains of the second helix.

The residues at position *d* (often leucine) contribute large hydrophobic stabilization energies, and are important for oligomer formation (Krystek: et al., *Int. J. Peptide Res.* 38:229, 1991). Lovejoy et al. (*Science* 259:1288, 1993) recently reported the synthesis of a triple-stranded  $\alpha$ -helical bundle in which the helices run up-up-down. Their studies confirmed that hydrophobic stabilization energy provides the main driving force for the formation of coiled coils from helical monomers. These studies also indicate that electrostatic interactions contribute to the stoichiometry and geometry of coiled coils. Further discussion of the structure of leucine zippers is found in Harbury et al. (*Science* 262:1401, 26 November 1993).

Examples of leucine zipper domains suitable for producing soluble oligomeric proteins are described in PCT application WO 94/10308, and the leucine zipper derived from lung surfactant protein D (SPD) described in Hoppe et al. (*FEBS Letters* 344:191, 1994), hereby incorporated by reference. The use of a modified leucine zipper that allows for stable trimerization of a heterologous protein fused thereto is described in Fanslow et al. (*Semin. Immunol.* 6:267-278, 1994). Recombinant fusion proteins comprising a soluble polypeptide fused to a leucine zipper peptide are expressed in suitable host cells, and the soluble oligomer that forms is recovered from the culture supernatant.

Certain leucine zipper moieties preferentially form trimers. One example is a leucine zipper derived from lung surfactant protein D (SPD), as described in Hoppe et al. (*FEBS Letters* 344:191, 1994)

and in U.S. Patent 5,716,805, hereby incorporated by reference in their entirety. This lung SPD-derived leucine zipper peptide comprises the amino acid sequence Pro Asp Val Ala Ser Leu Arg Gln Gln Val Glu Ala Leu Gln Gly Gln Val Gln His Leu Gln Ala Ala Phe Ser Gln Tyr.

Another example of a leucine zipper that promotes trimerization is a peptide comprising the amino acid sequence Arg Met Lys Gln Ile Glu Asp Lys Ile Glu Glu Ile Leu Ser Lys Ile Tyr His Ile Glu Asn Glu Ile Ala Arg Ile Lys Lys Leu Ile Gly Glu Arg, as described in U.S. Patent 5,716,805. In one alternative embodiment, an N-terminal Asp residue is added; in another, the peptide lacks the N-terminal Arg residue.

Fragments of the foregoing zipper peptides that retain the property of promoting oligomerization may be employed as well. Examples of such fragments include, but are not limited to, peptides lacking one or two of the N-terminal or C-terminal residues presented in the foregoing amino acid sequences. Leucine zippers may be derived from naturally occurring leucine zipper peptides, e.g., *via* conservative substitution(s) in the native amino acid sequence, wherein the peptide's ability to promote oligomerization is retained.

Other peptides derived from naturally occurring trimeric proteins may be employed in preparing trimeric IL-1 delta. Alternatively, synthetic peptides that promote oligomerization may be employed. In particular embodiments, leucine residues in a leucine zipper moiety are replaced by isoleucine residues. Such peptides comprising isoleucine may be referred to as isoleucine zippers, but are encompassed by the term "leucine zippers" as employed herein.

## PRODUCTION OF POLYPEPTIDES AND FRAGMENTS THEREOF

Expression, isolation and purification of the polypeptides and fragments of the invention may be accomplished by any suitable technique, including but not limited to the following:

### Expression Systems

The present invention also provides recombinant cloning and expression vectors containing DNA, as well as host cell containing the recombinant vectors. Expression vectors comprising DNA may be used to prepare the polypeptides or fragments of the invention encoded by the DNA. A method for producing polypeptides comprises culturing host cells transformed with a recombinant expression vector encoding the polypeptide, under conditions that promote expression of the polypeptide, then recovering the expressed polypeptides from the culture. The skilled artisan will recognize that the procedure for purifying the expressed polypeptides will vary according to such factors as the type of host cells employed, and whether the polypeptide is membrane-bound or a soluble form that is secreted from the host cell.

Any suitable expression system may be employed. The vectors include a DNA encoding a polypeptide or fragment of the invention, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences that control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a DNA sequence if the



promoter nucleotide sequence controls the transcription of the DNA sequence. An origin of replication that confers the ability to replicate in the desired host cells, and a selection gene by which transformants are identified, are generally incorporated into the expression vector.

In addition, a sequence encoding an appropriate signal peptide (native or heterologous) can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in frame to the nucleic acid sequence of the invention so that the DNA is initially transcribed, and the mRNA translated, into a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells promotes extracellular secretion of the polypeptide. The signal peptide is cleaved from the polypeptide upon secretion of polypeptide from the cell.

Suitable host cells for expression of polypeptides include prokaryotes, yeast or higher eukaryotic cells. Mammalian or insect cells are generally preferred for use as host cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce polypeptides using RNAs derived from DNA constructs disclosed herein.

#### Prokaryotic Systems

Prokaryotes include gram-negative or gram-positive organisms. Suitable prokaryotic host cells for transformation include, for example, *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include  $\beta$ -lactamase (penicillinase), lactose promoter system (Chang et al., *Nature* 275:615, 1978; and Goeddel et al., *Nature* 281:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res.* 8:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage  $\lambda$ <sub>P</sub> promoter and a c1857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the  $\lambda$ <sub>P</sub> promoter

include plasmid pHUB2 (resident in *E. coli* strain JMB9, ATCC 37092) and pPLc28 (resident in *E. coli* RRI, ATCC 53082).

IL-1 delta DNA may be cloned in-frame into the multiple cloning site of an ordinary bacterial expression vector. Ideally the vector would contain an inducible promoter upstream of the cloning site, such that addition of an inducer leads to high-level production of the recombinant protein at a time of the investigator's choosing. For some proteins, expression levels may be boosted by incorporation of codons encoding a fusion partner (such as hexahistidine) between the promoter and the gene of interest. The resulting "expression plasmid" may be propagated in a variety of strains of *E. coli*.

For expression of the recombinant protein, the bacterial cells are propagated in growth medium until reaching a pre-determined optical density. Expression of the recombinant protein is then induced, e.g. by addition of IPTG (isopropyl-b-D-thiogalactopyranoside), which activates expression of proteins from plasmids containing a lac operator/promoter. After induction (typically for 1-4 hours), the cells are harvested by pelleting in a centrifuge, e.g. at 5,000 x G for 20 minutes at 4°C.

For recovery of the expressed protein, the pelleted cells may be resuspended in ten volumes of 50 mM Tris-HCl (pH 8)/1 M NaCl and then passed two or three times through a French press. Most highly expressed recombinant proteins form insoluble aggregates known as inclusion bodies. Inclusion bodies can be purified away from the soluble proteins by pelleting in a centrifuge at 5,000 x G for 20 minutes, 4°C. The inclusion body pellet is washed with 50 mM Tris-HCl (pH 8)/1% Triton X-100 and then dissolved in 50 mM Tris-HCl (pH 8)/8 M urea/ 0.1 M DTT. Any material that cannot be dissolved is removed by centrifugation (10,000 x G for 20 minutes, 20°C). The protein of interest will, in most cases, be the most abundant protein in the resulting clarified supernatant. This protein may be "refolded" into the active conformation by dialysis against 50 mM Tris-HCl (pH 8)/5 mM CaCl<sub>2</sub>/5 mM Zn(OAc)<sub>2</sub>/1 mM GSSG/0.1 mM GSH. After refolding, purification can be carried out by a variety of chromatographic methods, such as ion exchange or gel filtration. In some protocols, initial purification may be carried out before refolding. As an example, hexahistidine-tagged fusion proteins may be partially purified on immobilized Nickel.

While the preceding purification and refolding procedure assumes that the protein is best recovered from inclusion bodies, those skilled in the art of protein purification will appreciate that many recombinant proteins are best purified out of the soluble fraction of cell lysates. In these cases, refolding is often not required, and purification by standard chromatographic methods can be carried out directly.

#### Yeast Systems

Alternatively, the polypeptides may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.* 255:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.* 7:149, 1968; and Holland et al., *Biochem.* 17:4900, 1978), such as enolase, glyceraldehyde-3-phosphate

dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phospho-glucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp<sup>r</sup> gene and origin of replication) into the above-described yeast vectors.

The yeast  $\alpha$ -factor leader sequence may be employed to direct secretion of the polypeptide. The  $\alpha$ -factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp<sup>+</sup> transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 mg/ml adenine and 20 mg/ml uracil.

Yeast host cells transformed by vectors containing an ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 mg/ml adenine and 80 mg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

#### Mammalian or Insect Systems

Mammalian or insect host cell culture systems also may be employed to express recombinant polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *BioTechnology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV1/EBNA cell line derived from the African green monkey kidney cell line CV1 (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Established methods for introducing DNA into mammalian cells have been described (Kaufman, R.J., *Large Scale Mammalian Cell Culture*, 1990, pp. 15-69). Additional protocols using commercially available reagents, such as Lipofectamine lipid reagent (Gibco/BRL) or Lipofectamine-Plus lipid reagent, can be used to transfect cells (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1987). In addition, electroporation can be used to transfect mammalian cells using conventional procedures, such as those in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, 1989). Selection of stable transformants can be performed using methods known in the art, such as,

for example, resistance to cytotoxic drugs. Kaufman et al., *Meth. in Enzymology* 185:487-511, 1990, describes several selection schemes, such as dihydrofolate reductase (DHFR) resistance. A suitable host strain for DHFR selection can be CHO strain DX-B11, which is deficient in DHFR (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77:4216-4220, 1980). A plasmid expressing the DHFR cDNA can be introduced into strain DX-B11, and only cells that contain the plasmid can grow in the appropriate selective media. Other examples of selectable markers that can be incorporated into an expression vector include cDNAs conferring resistance to antibiotics, such as G418 and hygromycin B. Cells harboring the vector can be selected on the basis of resistance to these compounds.

Transcriptional and translational control sequences for mammalian host cell expression vectors can be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from polyoma virus, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites can be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment, which can also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978; Kaufman, *Meth. in Enzymology*, 1990). Smaller or larger SV40 fragments can also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Additional control sequences shown to improve expression of heterologous genes from mammalian expression vectors include such elements as the expression augmenting sequence element (EASE) derived from CHO cells (Morris et al., *Animal Cell Technology*, 1997, pp. 529-534 and PCT Application WO 97/25420) and the tripartite leader (TPL) and VA gene RNAs from Adenovirus 2 (Gingras et al., *J. Biol. Chem.* 257:13475-13491, 1982). The internal ribosome entry site (IRES) sequences of viral origin allows dicistronic mRNAs to be translated efficiently (Oh and Sarnow, *Current Opinion in Genetics and Development* 3:295-300, 1993; Ramesh et al., *Nucleic Acids Research* 24:2697-2700, 1996). Expression of a heterologous cDNA as part of a dicistronic mRNA followed by the gene for a selectable marker (e.g. DHFR) has been shown to improve transfectability of the host and expression of the heterologous cDNA (Kaufman, *Meth. in Enzymology*, 1990). Exemplary expression vectors that employ dicistronic mRNAs are pTR-DC/GFP described by Mosser et al., *Biotechniques* 22:150-161, 1997, and p2A51 described by Morris et al., *Animal Cell Technology*, 1997, pp. 529-534.

A useful high expression vector, pCAVNOT, has been described by Mosley et al., *Cell* 59:335-348, 1989. Other expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature* 312:768, 1984, has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-A-0367566, and in WO 91/18982, incorporated by reference herein. In yet another alternative, the vectors can be derived from retroviruses.

Additional useful expression vectors, pFLAG<sup>®</sup> and pDC311, can also be used. FLAG<sup>®</sup> technology is centered on the fusion of a low molecular weight (1kD), hydrophilic, FLAG<sup>®</sup> marker peptide to the N-terminus of a recombinant protein expressed by pFLAG<sup>®</sup> expression vectors. pDC311 is another specialized vector used for expressing proteins in CHO cells. pDC311 is characterized by a bicistronic sequence containing the gene of interest and a dihydrofolate reductase (DHFR) gene with an internal ribosome binding site for DHFR translation, an expression augmenting sequence element (EASE), the human CMV promoter, a tripartite leader sequence, and a polyadenylation site.

Regarding signal peptides that may be employed, a heterologous signal peptide or leader sequence may be used, if desired. The choice of signal peptide or leader may depend on factors such as the type of host cells in which the recombinant polypeptide is to be produced. To illustrate, examples of heterologous signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

#### Purification

The invention also includes methods of isolating and purifying the polypeptides and fragments thereof. An isolated and purified IL-1 delta polypeptide according to the invention can be produced by recombinant expression systems as described above or purified from naturally occurring cells. IL-1 delta polypeptide can be substantially purified, as indicated by a single protein band upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). One process for producing IL-1 delta comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes IL-1 delta polypeptide under conditions sufficient to promote expression of IL-1 delta. IL-1 delta polypeptide is then recovered from culture medium or cell extracts, depending upon the expression system employed.

#### Isolation and Purification

The expression "isolated and purified" as used herein means that IL-1 delta is essentially free of association with other DNA, proteins, or polypeptides, for example, as a purification product of recombinant host cell culture or as a purified product from a non-recombinant source. The term "substantially purified" as used herein refers to a mixture that contains IL-1 delta and is essentially free of association with other DNA, proteins, or polypeptides, but for the presence of known DNA or proteins that can be removed using a specific antibody, and which substantially purified IL-1 delta proteins retain biological activity. The term "purified IL-1 delta" refers to either the "isolated and purified" form of IL-1 delta or the "substantially purified" form of IL-1 delta, as both are described herein.

The term "biologically active" as it refers to IL-1 delta protein, means that the IL-1 delta protein is capable of associating with IL-1 delta counterstructures or being co-immunoprecipitated with IL-1 delta counterstructures using an antibody to the IL-1 delta counterstructure.

In one preferred embodiment, the purification of recombinant polypeptides or fragments can be accomplished using fusions of polypeptides or fragments of the invention to another polypeptide to aid in the purification of polypeptides or fragments of the invention. Such fusion partners can include the poly-His or other antigenic identification peptides described above as well as the Fc moieties described previously.

With respect to any type of host cell, as is known to the skilled artisan, procedures for purifying a recombinant polypeptide or fragment will vary according to such factors as the type of host cells employed and whether or not the recombinant polypeptide or fragment is secreted into the culture medium.

In general, the recombinant polypeptide or fragment can be isolated from the host cells if not secreted, or from the medium or supernatant if soluble and secreted, followed by one or more concentration, salting-out, ion exchange, hydrophobic interaction, affinity purification or size exclusion chromatography steps. As to specific ways to accomplish these steps, the culture medium first can be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. In addition, a chromatofocusing step can be employed. Alternatively, a hydrophobic interaction chromatography step can be employed. Suitable matrices can be phenyl or octyl moieties bound to resins. In addition, affinity chromatography with a matrix which selectively binds the recombinant protein can be employed. Examples of such resins employed are lectin columns, dye columns, and metal-chelating columns. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel or polymer resin having pendant methyl, octyl, octyldecyl or other aliphatic groups) can be employed to further purify the polypeptides. Some or all of the foregoing purification steps, in various combinations, are well known and can be employed to provide an isolated and purified recombinant protein.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express IL-1 delta as a secreted polypeptide in order to simplify purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

It is also possible to utilize an affinity column comprising a polypeptide-binding protein of the invention, such as a monoclonal antibody generated against polypeptides of the invention, to affinity-purify expressed polypeptides. These polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized, or be competitively removed using the naturally occurring substrate of the affinity moiety, such as a polypeptide derived from the invention.

In this aspect of the invention, polypeptide-binding proteins, such as the anti-polypeptide antibodies of the invention or other proteins that may interact with the polypeptide of the invention, can be bound to a solid phase support such as a column chromatography matrix or a similar substrate suitable for identifying, separating, or purifying cells that express polypeptides of the invention on their surface. Adherence of polypeptide-binding proteins of the invention to a solid phase contacting surface can be accomplished by any means, for example, magnetic microspheres can be coated with these polypeptide-binding proteins and held in the incubation vessel through a magnetic field. Suspensions of cell mixtures are contacted with the solid phase that has such polypeptide-binding proteins thereon. Cells having polypeptides of the invention on their surface bind to the fixed polypeptide-binding protein and unbound cells then are washed away. This affinity-binding method is useful for purifying, screening, or separating such polypeptide-expressing cells from solution. Methods of releasing positively selected cells from the solid phase are known in the art and encompass, for example, the use of enzymes. Such enzymes are preferably non-toxic and non-injurious to the cells and are preferably directed to cleaving the cell-surface binding partner.

Alternatively, mixtures of cells suspected of containing polypeptide-expressing cells of the invention first can be incubated with a biotinylated polypeptide-binding protein of the invention. Incubation periods are typically at least one hour in duration to ensure sufficient binding to polypeptides of the invention. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides the binding of the polypeptide-binding cells to the beads. Use of avidin-coated beads is known in the art. See Berenson, et al. *J. Cell. Biochem.*, 10D:239 (1986). Wash of unbound material and the release of the bound cells is performed using conventional methods.

In the methods described above, suitable IL-1 delta-binding polypeptides are anti-IL-1 delta antibodies and other proteins that are capable of high-affinity binding of IL-1 delta. A preferred IL-1 delta-binding protein is an anti-IL-1 delta monoclonal antibody.

The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the polypeptide is to be administered *in vivo*, for example. In such a case, the polypeptides are purified such that no protein bands corresponding to other proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to the polypeptide may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. Most preferably, the polypeptide of the invention is purified to substantial homogeneity, as indicated by a single protein band

upon analysis by SDS-PAGE. The protein band may be visualized by silver staining, Coomassie blue staining, or (if the protein is radiolabeled) by autoradiography.

#### Assays

The purified polypeptides of the invention (including proteins, polypeptides, fragments, variants, oligomers, and other forms) may be tested for the ability to bind a IL-1 delta counter-structure molecule in any suitable assay, such as a conventional binding assay. To illustrate, the polypeptide may be labeled with a detectable reagent (e.g., a radionuclide, chromophore, enzyme that catalyzes a colorimetric or fluorometric reaction, and the like). The labeled polypeptide is contacted with cells expressing a IL-1 delta counter-structure molecule. The cells then are washed to remove unbound labeled polypeptide, and the presence of cell-bound label is determined by a suitable technique, chosen according to the nature of the label.

One example of a binding assay procedure is as follows. A recombinant expression vector containing a IL-1 delta counter-structure molecule cDNA is constructed, for example, fusing the extracellular domain of a IL-1 delta counter-structure molecule to the IgG-I Fc (mucin form) as previously described for OX40-Fc (Baum et al., *EMBO J.* 13:3992-4001, 1994). CV1-EBNA-1 cells in 10 cm<sup>2</sup> dishes are transfected with the recombinant expression vector. CV-1/EBNA-1 cells (ATCC CRL 10478) constitutively express EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

The transfected cells are cultured for 24 hours, and the cells in each dish then are split into a 24-well plate. After culturing an additional 48 hours, the transfected cells (about  $4 \times 10^4$  cells/well) are washed with BM-NFDM, which is binding medium (RPMI 1640 containing 25 mg/ml bovine serum albumin, 2 mg/ml sodium azide, 20 mM Hepes pH 7.2) to which 50 mg/ml nonfat dry milk has been added. The cells then are incubated for 1 hour at 37°C with various concentrations of, for example, a soluble polypeptide/Fc fusion protein made as set forth above. Cells then are washed and incubated with a constant saturating concentration of a <sup>125</sup>I-mouse anti-human IgG in binding medium, with gentle agitation for 1 hour at 37°C. After extensive washing, cells are released *via* trypsinization.

The mouse anti-human IgG employed above is directed against the Fc region of human IgG and can be obtained from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA. The antibody is radioiodinated using the standard chloramine-T method. The antibody will bind to the Fc portion of any polypeptide/Fc protein that has bound to the cells. In all assays, non-specific binding of <sup>125</sup>I-antibody is assayed in the absence of the Fc fusion protein, as well as in the presence of the Fc fusion protein and a 200-fold molar excess of unlabeled mouse anti-human IgG antibody.

Cell-bound <sup>125</sup>I-antibody is quantified on a Packard Autogamma counter. Affinity calculations (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) are generated on RS/1 (BBN Software, Boston, MA) run on a Microvax computer.



Another type of suitable binding assay is a competitive binding assay. To illustrate, biological activity of a variant may be determined by assaying for the variant's ability to compete with the native protein for binding to IL-1 delta counterstructures or cells expressing a IL-1 delta counterstructure.

Competitive binding assays can be performed by conventional methodology. Reagents that may be employed in competitive binding assays include radiolabeled IL-1 delta and intact cells expressing IL-1 delta counterstructures (endogenous or recombinant) on the cell surface. For example, a radiolabeled soluble IL-1 delta fragment can be used to compete with a soluble IL-1 delta variant for binding to cell surface (binding partner). Instead of intact cells, one could substitute a soluble IL-1 delta counterstructure/Fc fusion protein bound to a solid phase through the interaction of Protein A or Protein G (on the solid phase) with the Fc moiety. Chromatography columns that contain Protein A and Protein G include those available from Pharmacia Biotech, Inc., Piscataway, NJ.

Another type of competitive binding assay utilizes radiolabeled soluble IL-1 delta counterstructure such as a soluble IL-1 delta counterstructure/Fc fusion protein, and intact cells expressing IL-1 delta. Qualitative results can be obtained by competitive autoradiographic plate binding assays, while Scatchard plots (Scatchard, *Ann. N.Y. Acad. Sci.* 51:660, 1949) may be utilized to generate quantitative results.

#### **USE OF IL-1 delta NUCLEIC ACID OR OLIGONUCLEOTIDES**

In addition to being used to express polypeptides as described above, the nucleic acids of the invention, including DNA, and oligonucleotides thereof can be used:

- as probes to identify nucleic acid encoding proteins having IL-1 delta activity;
- to identify human chromosome 2;
- to map genes on human chromosome 2;
- to identify genes associated with certain diseases, syndromes, or other conditions associated with human chromosome 2;
- as single-stranded sense or antisense oligonucleotides, to inhibit expression of polypeptide encoded by the IL-1 delta gene;
- to help detect defective genes in an individual; and
- for gene therapy.

#### **Probes**

Among the uses of nucleic acids of the invention is the use of fragments as probes or primers. Such fragments generally comprise at least about 17 contiguous nucleotides of a DNA sequence. In other embodiments, a DNA fragment comprises at least 30, or at least 60, contiguous nucleotides of a DNA sequence.

Because homologs of SEQ ID NO:1 and SEQ ID NO:3 from other mammalian species are contemplated herein, probes based on the DNA sequence of SEQ ID NO:1 or SEQ ID NO:3 may be used to screen cDNA libraries derived from other mammalian species, using conventional cross-species hybridization techniques.

Using knowledge of the genetic code in combination with the amino acid sequences set forth above, sets of degenerate oligonucleotides can be prepared. Such oligonucleotides are useful as primers, e.g., in polymerase chain reactions (PCR), whereby DNA fragments are isolated and amplified.

Due to the RNA expression pattern of IL-1 delta, probes based on the DNA sequence of SEQ ID NO:3 can be used to detect lymph node, thymus, tonsil, brain placenta, lung, skeletal muscle, prostate, and testis tissue and cell types by methods such as *in situ* hybridization.

#### Chromosome Mapping

Human IL-1 delta gene maps to chromosome 2q11-12. All or a portion of the nucleic acids of SEQ ID NO:3, including oligonucleotides, can be used by those skilled in the art using well-known techniques to identify human chromosome 2, and the specific locus thereof, that contains the DNA of IL-1 delta family members. Useful techniques include, but are not limited to, using the sequence or portions, including oligonucleotides, as a probe in various well-known techniques such as radiation hybrid mapping (high resolution), *in situ* hybridization to chromosome spreads (moderate resolution), and Southern blot hybridization to hybrid cell lines containing individual human chromosomes (low resolution).

For example, chromosomes can be mapped by radiation hybridization.

PCR is performed using the Whitehead Institute/MIT Center for Genome Research Genebridge4 panel of 93 radiation hybrids ([http://www-genome.wi.mit.edu/ftp/distribution/human\\_STS\\_releases/july97/rhmap/genebridge4.html](http://www-genome.wi.mit.edu/ftp/distribution/human_STS_releases/july97/rhmap/genebridge4.html)). Primers are used which lie within a putative exon of the gene of interest and which amplify a product from human genomic DNA, but do not amplify hamster genomic DNA. The results of the PCRs are converted into a data vector that is submitted to the Whitehead/MIT Radiation Mapping site on the internet (<http://www-seq.wi.mit.edu>). The data is scored and the chromosomal assignment and placement relative to known Sequence Tag Site (STS) markers on the radiation hybrid map is provided. The following web site provides additional information about radiation hybrid mapping: [http://www-genome.wi.mit.edu/ftp/distribution/human\\_STS\\_releases/july97/07-97.INTRO.html](http://www-genome.wi.mit.edu/ftp/distribution/human_STS_releases/july97/07-97.INTRO.html)).

#### Identifying Associated Diseases

As set forth above, SEQ ID NO:3 has been mapped to the 2q11-12 region of chromosome 2.

Human chromosome 2 is associated with specific diseases which include but are not limited to glaucoma, ectodermal dysplasia, insulin-dependent diabetes mellitus, wrinkly skin syndrome, T-cell leukemia/lymphoma, and tibial muscular dystrophy. Thus, the nucleic acid of SEQ ID NO:3, or a fragment thereof, can be used by one skilled in the art using well-known techniques to analyze abnormalities associated with gene mapping to chromosome 2. This enables one to distinguish conditions in which this marker is rearranged or deleted. In addition, nucleotides of SEQ ID NO:3 or a fragment thereof can be used as a positional marker to map other genes of unknown location.

The DNA may be used in developing treatments for any disorder mediated (directly or indirectly) by defective, or insufficient amounts of, the genes corresponding to the nucleic acids of the invention. Disclosure herein of native nucleotide sequences permits the detection of defective genes, and the

replacement thereof with normal genes. Defective genes may be detected in *in vitro* diagnostic assays, and by comparison of a native nucleotide sequence disclosed herein with that of a gene derived from a person suspected of harboring a defect in this gene.

#### Sense-Antisense

Other useful fragments of the nucleic acids include antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target mRNA (sense) or DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of DNA (SEQ ID NO:1 or SEQ ID NO:3). Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen (*Cancer Res.* 48:2659, 1988) and van der Krol et al. (*BioTechniques* 6:958, 1988).

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block or inhibit protein expression by one of several means, including enhanced degradation of the mRNA by RNaseH, inhibition of splicing, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oligonucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, lipofection, CaPO<sub>4</sub>-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus.

Sense or antisense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the sense or antisense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO

91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase. to a monoclonal antibody targeted to a specific cell type.

#### **USE OF IL-1 delta POLYPEPTIDES AND POLYPEPTIDES**

Uses include, but are not limited to, the following:

- Purifying proteins and measuring activity thereof
- Delivery Agents
- Therapeutic Agents
- Rational Drug Design
- Research Reagents
- Preparation of Antibodies

#### **Purification Reagents**

Each of the polypeptides of the invention finds use as a protein purification reagent. The polypeptides may be attached to a solid support material and used to purify IL-1 delta counter-structure molecules by affinity chromatography. In particular embodiments, a polypeptide (in any form described herein that is capable of binding IL-1 delta counter-structure molecules) is attached to a solid support by conventional procedures. As one example, chromatography columns containing functional groups that will react with functional groups on amino acid side chains of proteins are available (Pharmacia Biotech, Inc., Piscataway, NJ). In an alternative, a polypeptide/Fc protein (as discussed above) is attached to Protein A- or Protein G-containing chromatography columns through interaction with the Fc moiety.

The polypeptide also finds use in purifying or identifying cells that express IL-1 delta counter-structure molecules on the cell surface. Polypeptides are bound to a solid phase such as a column chromatography matrix or a similar suitable substrate. For example, magnetic microspheres can be coated with the polypeptides and held in an incubation vessel through a magnetic field. Suspensions of cell mixtures containing IL-1 delta counter-structure molecule- expressing cells are contacted with the solid phase having the polypeptides thereon. Cells expressing IL-1 delta counter-structure molecules on the cell surface bind to the fixed polypeptides, and unbound cells then are washed away.

Alternatively, the polypeptides can be conjugated to a detectable moiety, then incubated with cells to be tested for IL-1 delta counter-structure molecules expression. After incubation, unbound labeled matter is removed and the presence or absence of the detectable moiety on the cells is determined.

In a further alternative, mixtures of cells suspected of containing cells expressing IL-1 delta counter-structure molecules are incubated with biotinylated polypeptides. Incubation periods are typically at least one hour in duration to ensure sufficient binding. The resulting mixture then is passed through a column packed with avidin-coated beads, whereby the high affinity of biotin for avidin provides binding of the desired cells to the beads. Procedures for using avidin-coated beads are known (see Berenson, et al. *J. Cell. Biochem.*, 10D:239, 1986). Washing to remove unbound material, and the release of the bound cells, are performed using conventional methods.

#### Measuring Activity

Polypeptides also find use in measuring the biological activity of IL-1 delta counter-structure molecules in terms of their binding affinity. The polypeptides thus may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of protein under different conditions. For example, the polypeptides may be employed in a binding affinity study to measure the biological activity of a IL-1 delta counter-structure molecule that has been stored at different temperatures, or produced in different cell types. The proteins also may be used to determine whether biological activity is retained after modification of a IL-1 delta counter-structure molecule (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified IL-1 delta counter-structure molecule is compared to that of an unmodified IL-1 delta counter-structure molecule to detect any adverse impact of the modifications on biological activity of IL-1 delta counter-structure molecules. The biological activity of a IL-1 delta counter-structure molecule thus can be ascertained before it is used in a research study, for example.

#### Delivery Agents

The polypeptides can be used to deliver diagnostic or therapeutic agents to such cells or cell types found to express IL-1 delta counterstructure molecules on the cell surface in *in vitro* or *in vivo* procedures. Therefore, IL-1 delta polypeptide can be attached to a toxin to bind to cells that express IL-1 delta counterstructure molecules on the cell surface and specifically kill these cells. The methodology can be similar to the successful use of an anti-CD72 immunotoxin to treat therapy-refractory B-lineage acute lymphoblastic leukemia in SCID mice (Meyers et al., *Leuk. and Lymph.* 18:119-122).

Detectable (diagnostic) and therapeutic agents that may be attached to a polypeptide include, but are not limited to, toxins, other cytotoxic agents, drugs, radionuclides, chromophores, enzymes that catalyze a colorimetric or fluorometric reaction, and the like, with the particular agent being chosen according to the intended application. Among the toxins are ricin, abrin, diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A, ribosomal inactivating proteins, mycotoxins such as trichothecenes, and derivatives and fragments (e.g., single chains) thereof. Radionuclides suitable for diagnostic use include, but are not limited to,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{111}\text{In}$ , and  $^{76}\text{Br}$ . Examples of radionuclides suitable for therapeutic use are  $^{131}\text{I}$ ,  $^{211}\text{At}$ ,  $^{77}\text{Br}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{212}\text{Pb}$ ,  $^{212}\text{Bi}$ ,  $^{109}\text{Pd}$ ,  $^{64}\text{Cu}$ , and  $^{67}\text{Cu}$ .

Such agents may be attached to the polypeptide by any suitable conventional procedure. The polypeptide comprises functional groups on amino acid side chains that can be reacted with functional groups on a desired agent to form covalent bonds, for example. Alternatively, the protein or agent may be

derivatized to generate or attach a desired reactive functional group. The derivatization may involve attachment of one of the bifunctional coupling reagents available for attaching various molecules to proteins (Pierce Chemical Company, Rockford, Illinois). A number of techniques for radiolabeling proteins are known. Radionuclide metals may be attached to polypeptides by using a suitable bifunctional chelating agent, for example.

Conjugates comprising polypeptides and a suitable diagnostic or therapeutic agent (preferably covalently linked) are thus prepared. The conjugates are administered or otherwise employed in an amount appropriate for the particular application.

#### Therapeutic Agents

Another embodiment of the invention relates to therapeutic uses of IL-1 delta. IL-1 family members play a central role in protection against infection and immune inflammatory responses which includes cellular signal transduction, activating vascular endothelial cells and lymphocytes, induction of inflammatory cytokines, acute phase proteins, hematopoiesis, fever, bone resorption, prostaglandins, metalloproteinases, and adhesion molecules. With the continued increase in the number of known IL-1 family members, a suitable classification scheme is one based on comparing polypeptide structure as well as function (activation and regulatory properties). Thus, the receptor for IL-1 delta, like those for IL-1  $\alpha$ , IL-1  $\beta$ , and IL-18, would likely be involved in many of the functions noted above and therefore perhaps be involved in the causation and maintenance of inflammatory and/or autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and psoriasis. As such, alterations in the expression and/or activation of antagonist IL-1 family members such as IL-1ra and IL-1 delta can have profound effects on a plethora of cellular processes, including, but not limited to, activation or inhibition of cell specific responses, proliferation, and inflammatory reactions based on changes in signal transduction.

IL-1 mediated cellular signaling often involves a molecular activation cascade, during which a receptor propagates a ligand-receptor mediated signal by specifically activating intracellular kinases which phosphorylate target substrates, resulting in the activation of the transcription factors NFkappaB and AP1, the protein kinases Jun N-terminal kinase and p38 map kinase, the enzymes COX-2 leading to prostaglandin production and iNOS leading to nitric oxide production, and inflammation in general. Therefore, administration of IL-1 is useful in circumstances where an increase in nitric oxide, adhesion molecules, JNK and p38 MAP kinase activity, etc. would be helpful, e.g. to stimulate an immune or inflammatory response. Accordingly, IL-1 has therapeutic uses, such as protecting against infection and generating immune and inflammatory responses in individuals, including those whose immune and/or inflammatory responses are inappropriate or nonresponsive. For example, IL-1 is useful in stimulating the immune system of individuals whose immune system is immunosuppressed.

One IL-1 family member, IL-1 receptor antagonist (IL-1ra), acts as an antagonist of the actions of IL-1 alpha and IL-1beta. Moreover, as described herein, IL-1 delta exhibits the highest degree of similarity to IL-1ra as compared to any other IL-1 family members. Therefore, administration of IL-1 delta will have therapeutic application in blocking inflammatory responses, including the activation of transcription factors NFkappaB and AP1, the protein kinases Jun N-terminal kinase and p38 MAP kinase, the enzymes COX-2

leading to prostaglandin production and iNOS leading to nitric oxide production, and inflammation in general.

Similarly, because IL-1 promotes inflammatory responses and is involved in the causation and maintenance of inflammatory and/or autoimmune diseases, antagonists of IL-1 (for example, IL-1 delta) are useful in inhibiting or treating inflammatory and/or autoimmune disease. Thus, IL-1 delta will be useful in treating arthritic conditions that have an inflammatory or autoimmune component, for example, rheumatoid arthritis and/or ankylosing spondylitis; inflammatory bowel disease, including Crohn's Disease and ulcerative colitis, and psoriasis (including psoriatic arthritis). Other inflammatory and/or autoimmune diseases in which IL-1 is implicated include pulmonary conditions relating to an immune or inflammatory response and/or in which airway hyperreactivity plays a role, for example, asthma, infection-associated airway hyperactivity, granulomatous lung disease, emphysema and chronic fibrosing alveolitis and acute hyperoxic lung damage, and demyelinating conditions that have an inflammatory or autoimmune component, for example, multiple sclerosis and/or chronic inflammatory demyelinating polyneuropathy. IL-1 delta will also be useful in ameliorating these conditions.

Additional conditions for which an autoimmune and/or inflammatory component is a contributory factor (and thus, for which antagonists of IL-1 such as IL-1 delta are useful) include cardiovascular conditions such as stroke, acute myocardial infarction, unstable angina, arterial restenosis and congestive heart failure. IL-1 antagonists (i.e., IL-1 delta) are useful in treating or preventing osteoporosis and/or osteoarthritis, as well as glomerulonephritis, uveitis, and/or Behçet's syndrome. An autoimmune or inflammatory component also plays a role in the cause or maintenance of sepsis, acute pancreatitis, diabetes (particularly Type II, insulin dependent diabetes), endometriosis, and periodontal disease. Similarly, the inflammatory response causes or exacerbates heart stroke and glaucoma, and the cytokines involved in the immune/inflammatory response play a supportive role in neoplastic disease (for example, in multiple myeloma and/or myeloid leukemia), facilitating the growth of neoplastic cells. Accordingly, IL-1 delta is useful in treating or ameliorating these conditions by downregulating the immune and/or inflammatory response that plays a causative role therein.

Polypeptides (or other moieties) can be introduced into the extracellular environment by well-known means, such as by administering the protein intravenously or coupling it to a monoclonal antibody targeted to a specific cell type, to thereby affect signaling. When used as a therapeutic agent, polypeptides of the invention can be formulated into pharmaceutical compositions according to known methods. The polypeptides can be combined in admixture, either as the sole active material or with other known active materials, with pharmaceutically suitable diluents (e.g., Tris-HCl, acetate, phosphate), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed. 1980, Mack Publishing Co. In addition, such compositions can contain the polypeptides complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of polypeptides of the invention.

The dosage of the composition can be readily determined by those of ordinary skill in the art. The amount to be administered and the frequency of administration can be determined empirically and will take into consideration the age and size of the patient being treated, as well as the malady being treated.

Treatment comprises administering the composition by any method familiar to those of ordinary skill in the art, including intravenous, intraperitoneal, intracorporeal injection, intra-articular, intraventricular, intrathecal, intramuscular, subcutaneous, topically, tonsillar, intranasally, intravaginally, and orally. The composition may also be given locally, such as by injection into the particular area, either intramuscularly or subcutaneously.

#### Drug Discovery

The purified polypeptides according to the invention will facilitate the discovery of inhibitors (or antagonists) and/or agonists of such polypeptides. The use of a purified polypeptide of the invention in the screening of potential inhibitors and/or agonists thereof is important and can eliminate or reduce the possibility of interfering reactions with contaminants.

In addition, polypeptides of the invention can be used for structure-based design of polypeptide-inhibitors and/or agonists. Such structure-based design is also known as "rational drug design." The polypeptides can be three-dimensionally analyzed by, for example, X-ray crystallography, nuclear magnetic resonance or homology modeling, all of which are well-known methods. The use of the polypeptide structural information in molecular modeling software systems to assist in inhibitor design and inhibitor-polypeptide interaction is also encompassed by the invention. Such computer-assisted modeling and drug design can utilize information such as chemical conformational analysis, electrostatic potential of the molecules, protein folding, etc. For example, most of the design of class-specific inhibitors of metalloproteases has focused on attempts to chelate or bind the catalytic zinc atom. Synthetic inhibitors are usually designed to contain a negatively-charged moiety to which is attached a series of other groups designed to fit the specificity pockets of the particular protease. A particular method of the invention comprises analyzing the three dimensional structure of polypeptides of the invention for likely binding sites of substrates, synthesizing a new molecule that incorporates a predictive reactive site, and assaying the new molecule as described above.

Specific screening methods are known in the art and along with integrated robotic systems and collections of chemical compounds/natural products are extensively incorporated in high throughput screening so that large numbers of test compounds can be tested for antagonist or agonist activity within a short amount of time. These methods include homogeneous assay formats such as fluorescence resonance energy transfer, fluorescence polarization, time-resolved fluorescence resonance energy transfer, scintillation proximity assays, reporter gene assays, fluorescence quenched enzyme substrate, chromogenic enzyme substrate and electrochemiluminescence, as well as more traditional heterogeneous assay formats such as enzyme-linked immunosorbent assays (ELISA) or radioimmunoassays. Homogeneous assays are preferred. Also comprehended herein are cell-based assays, for example those utilizing reporter genes, as well as functional assays that analyze the effect of an antagonist or agonist on biological function(s) or activity(ies) of IL-1 delta (for example, stimulation of the secretion of cytokines or inhibition thereof, as



disclosed herein). Moreover, animal models of inflammatory conditions are useful assays of biological activity.

Accordingly, in one aspect of the invention, there is provided a method for screening a test compound to determine whether the test compound affects (or modulates) a biological activity of an IL-1 delta polypeptide, the method comprising contacting the test compound and the IL-1 delta polypeptide with cells capable of exhibiting the biological activity when contacted with IL-1 delta, and analyzing the cells for the occurrence of the biological activity, wherein if the biological activity observed in the presence of the test compound differs from the biological activity that is observed when the test compound is absent, the test compound affects the biological activity of the IL-1 delta. The cells may be contacted *in vitro* or *in vivo*.

As used herein, the IL-1 delta polypeptide comprises a polypeptide selected from the group consisting of the polypeptides of SEQ ID NO:2, and SEQ ID NO:4, and polypeptides encoded by DNAs that hybridize under moderately stringent conditions to the DNAs of SEQ ID NO:1 or SEQ ID NO:3. Such polypeptides include polypeptides comprising variant amino acid sequences that are at least 80% identical to the polypeptides of SEQ ID NO:2 or SEQ ID NO:4 (preferably, the variant amino acid sequences that are at least 90% identical, more preferably at least 95% identical, most preferably at least 97% identical, to the polypeptides of SEQ ID NO:2 or SEQ ID NO:4). Additional examples of useful IL-1 delta polypeptides include polypeptides comprising the amino acid sequences of SEQ ID NOs:2 or 4 wherein the polypeptides comprise alterations to the amino acid sequences selected from the group consisting of inactivated N-glycosylation site(s), inactivated protease processing site(s), conservative amino acid substitution(s), and combinations thereof. Moreover, fragments of the aforesaid polypeptides that have at least one activity of IL-1 delta as described below are also comprehended herein.

IL-1 delta biological activity includes, but is not limited to, modulation of cytokine expression, modulation of the expression of molecules indicative of activation of an immune or inflammatory response (for example, COX2, iNOS), modulation of cell-surface molecule expression, modulation of activation of one or more signaling cascades, modulation of induction of mRNAs for the aforementioned proteins, modulation of induction of cell proliferation and/or cell death, induction of morphological and/or functional changes in cells, and combinations thereof. The inventive methods comprise methods of assaying for any of these biological activities. Those of skill in the art will recognize that modulation of cytokines means that the levels of expression of certain cytokines increase while the levels of other cytokines decreases, and that such combinations are comprehended in the term modulation; the same is true for other activities of IL-1 delta.

When the methods of the present invention include assaying for IL-1 delta modulation of cytokine expression, cytokines that may be assayed include (but are not limited to) IL-1 alpha, IL-1 beta, TNF-alpha, IL-10, IFN-gamma, IL-12 (in particular, the p40 subunit), IL-6, IL-1ra, IL-4, IL-13, GM-CSF, IL-18, IL-1 homologs such as IL-1 epsilon, IL-1 eta, IL-1 theta, IL-1 zeta, and IL-1 H1, and combinations thereof. Similarly, when the screening methods of the present invention include assaying for IL-1 delta modulation of cell surface molecule expression, the cell surface molecules that may be assayed include ICAM-1, TLR4,

TLR5, TLR9, DC-B7, MHC class I and II antigens, VCAM, ELAM, B7-1, B7-2, CD40L, and combinations thereof.

IL-1 delta mediated modulation of signaling pathways often involves a cascade of molecular changes, for example as discussed previously wherein a receptor propagates a ligand-receptor mediated signal by specifically activating intracellular kinases which phosphorylate target substrates (which can themselves be kinases that become activated following phosphorylation, or adaptor molecules that facilitate down-stream signaling through protein-protein interaction following phosphorylation), resulting in the activation of other factors (for example, NFkappaB). When the screening methods of the present invention include assaying for IL-1 delta induced modulation of signaling pathways, the signaling pathways that may be assayed include those involving activation of NFkappaB. Assaying for activation signaling cascades further includes detecting phosphorylation of molecules that occurs during the signaling cascade, as in the phosphorylation of IkappaB (including IkappaB degradation assays, and assays for free IkappaB), p38 MAP kinase, and Stress-Activated Protein Kinase (SAPK/JNK).

Moreover, those of skill in the art understand that biological activity(ies) is/are most often induced by the binding of a ligand (i.e., IL-1) to a receptor (counterstructure or binding moiety) present on a cell. IL-1 delta, like IL-1ra, binds a receptor and inhibits binding of a ligand thereto; accordingly, as previously described, IL-1 delta polypeptides (including IL-1 delta polypeptide fragments) can be used in binding studies to identify receptor-expressing cells. Such binding studies also provide assays useful in the inventive methods. IL-1 delta polypeptides may also be used to clone receptors (or other molecules that bind IL-1 delta) and to screen for molecules that block receptor/ligand interactions. Those of ordinary skill in the art further understand that biological activities include cell proliferation, cell death, and changes in cell morphology and/or function (for example, activation, maturation); assays that evaluate such effects of IL-1 delta are known in the art, and will also be useful in the inventive methods. Moreover, animal models of syndromes and/or conditions, such as those disclosed herein, are useful for screening compounds for biological activity, including screening for antagonism (or agonism) of IL-1 delta.

The inventive methods further encompass performing more than one assay to discover and/or analyze agonists or antagonists of IL-1 delta activity (i.e., combination methods). Generally, such methods comprise selecting test compounds that affect a property of IL-1 delta (i.e., an ability of IL-1 delta to bind an IL-1 delta counter structure), then testing the selected compounds for an effect on another property of IL-1 delta (i.e., contacting the selected test compounds and an IL-1 delta polypeptide with cells capable of exhibiting a biological activity when contacted with IL-1 delta, and determining whether the compounds affect the biological activity). For example, the inventive methods may comprise a first assay to determine whether a candidate molecule interacts with (binds to) IL-1 delta. In one embodiment, the first assay is in a high throughput format, numerous forms of which are known in the art and disclosed herein. Such an assay will generally comprise the steps of: contacting test compounds and an IL-1 delta polypeptide with an IL-1 delta counterstructure; determining whether the test compounds affect the ability of IL-1 delta to bind the counterstructure; and selecting one or more test compounds that affect the ability of IL-1 delta to bind the counterstructure. The inventive combination methods further comprise evaluating selected compounds in a

second assay, for agonistic or antagonistic effect on biological activity using one or more of the aforementioned assays.

Alternatively, the inventive combination methods may comprise a first assay to determine whether a candidate molecule modulates a biological activity of IL-1 delta, as described herein using an *in vitro* assay or an *in vivo* assay (for example, an animal model). According to such combination methods, molecules that modulate an IL-1 delta biological activity in this manner are selected using one or more of the aforementioned assays for biological activity, and assayed to determine whether the candidate molecule(s) bind IL-1 delta. The selected molecules may be tested to further define the exact region or regions of IL-1 delta to which the test molecule binds (for example, epitope mapping for antibodies).

As disclosed previously, the types of assays for biological activities of IL-1 delta that can be used in the inventive combination methods include assays for the expression of cytokines, assays for the expression of cell-surface molecules, assays to detect activation of signaling molecules, assays to detect induction of mRNAs, and assays that evaluate cell proliferation or cell death (and combinations thereof), as described herein. Molecules that bind and that have an agonistic or antagonistic effect on biologic activity will be useful in treating or preventing diseases or conditions with which the polypeptide(s) are implicated.

Those of ordinary skill in the art understand that when the biological activity observed in the presence of the test compound is greater than that observed when the test compound is absent, the test compound is an agonist of IL-1 delta, whereas when the biological activity observed in the presence of the test compound is less than that observed when the test compound is absent, the test compound is an antagonist (or inhibitor) of IL-1 delta. Generally, an antagonist will decrease or inhibit, an activity by at least 30%; more preferably, antagonists will inhibit activity by at least 50%, most preferably by at least 90%. Similarly, an agonist will increase, or enhance, an activity by at least 20%; more preferably, agonists will enhance activity by at least 30%, most preferably by at least 50%. Those of skill in the art will also recognize that agonists and/or antagonists with different levels of agonism or antagonism respectively may be useful for different applications (i.e., for treatment of different disease states).

Homogeneous assays are mix-and-read style assays that are very amenable to robotic application, whereas heterogeneous assays require separation of free from bound analyte by more complex unit operations such as filtration, centrifugation or washing. These assays are utilized to detect a wide variety of specific biomolecular interactions (including protein-protein, receptor-ligand, enzyme-substrate, and so on), and the inhibition thereof by small organic molecules. These assay methods and techniques are well known in the art (see, e.g., *High Throughput Screening: The Discovery of Bioactive Substances*, John P. Devlin (ed.), Marcel Dekker, New York, 1997 ISBN: 0-8247-0067-8). The screening assays of the present invention are amenable to high throughput screening of chemical libraries and are suitable for the identification of small molecule drug candidates, antibodies, peptides, and other antagonists and/or agonists, natural or synthetic. Several useful assays are disclosed in USSN 09/851,673, filed May 8, 2001 (the relevant disclosure of which is hereby incorporated by reference).

Candidate Molecules to be Tested:

The methods of the invention may be used to identify antagonists (also referred to as inhibitors) and agonists of IL-1 delta activity from cells, cell-free preparations, chemical libraries, cDNA libraries, recombinant antibody libraries (or libraries comprising subunits of antibodies) and natural product mixtures. The antagonists and agonists may be natural or modified substrates, ligands, enzymes, receptors, etc. of the polypeptides of the instant invention, or may be structural or functional mimetics of IL-1 delta or its binding partner/counterstructure. Potential antagonists of the instant invention include small molecules, peptides and antibodies that bind to and occupy a binding site of the inventive polypeptides or a binding partner thereof, causing them to be unavailable to bind to their natural binding partners and therefore preventing normal biological activity. Antagonists also include chemicals (including small molecules and peptides) that interfere with the signaling pathways used by IL-1 delta (for example, by inhibiting the interaction of receptor subunits, or inhibiting the interaction of intracellular components of the signaling cascade). Potential agonists include small molecules, peptides and antibodies which bind to the instant polypeptides or binding partners thereof, and elicit the same or enhanced biologic effects as those caused by the binding of the polypeptides of the instant invention. Moreover, substances that activate (or enhance) the signaling pathways used by IL-1 delta are also included within the scope of agonists of IL-1 delta.

Small molecule agonists and antagonists are usually less than 10K molecular weight and may possess a number of physicochemical and pharmacological properties which enhance cell penetration, resist degradation and prolong their physiological half-lives (Gibbs, J., *Pharmaceutical Research in Molecular Oncology*, Cell, Vol. 79 (1994)). Antibodies, which include intact molecules as well as fragments such as Fab and F(ab')<sub>2</sub> fragments, as well as recombinant molecules derived therefrom (including antibodies expressed on phage, intrabodies, single chain antibodies such as scFv and other molecules derived from immunoglobulins that are known in the art), may be used to bind to and inhibit the polypeptides of the instant invention by blocking the propagation of a signaling cascade. It is preferable that the antibodies are humanized, and more preferable that the antibodies are human. The antibodies of the present invention may be prepared by any of a variety of well-known methods, as disclosed herein.

Additional examples of candidate molecules, also referred to herein as "test molecules" or "test compounds," to be tested for the ability to modulate IL-1 delta activity include, but are not limited to, carbohydrates, small molecules (usually organic molecules or peptides), proteins, and nucleic acid molecules (including oligonucleotide fragments typically consisting of from 8 to 30 nucleic acid residues). Peptides to be tested typically consist of from 5 to 25 amino acid residues. Also, candidate nucleic acid molecules can be antisense nucleic acid sequences, and/or can possess ribozyme activity.

Small molecules to be screened using the hereindescribed screening assays can typically be administered orally or by injection to a patient in need thereof. Small molecules that can be administered orally are especially preferred. The small molecules of the invention preferably will not be toxic (or only minimally toxic) at the doses required for them to be effective as pharmaceutical agents, and they are preferably not subject to rapid loss of activity in the body, such as the loss of activity that might result from rapid enzymatic or chemical degradation. In addition, pharmaceutically useful small molecules are preferably not immunogenic.

The methods of the invention can be used to screen for antisense molecules that inhibit the functional expression of one or more mRNA molecules that encode one or more proteins that mediate an IL-1 delta-dependent cellular response. An anti-sense nucleic acid molecule is a DNA sequence that is capable of hybridizing to the target mRNA molecule through Watson-Crick base pairing, and inhibiting translation thereof. Alternatively, the DNA may be inverted relative to its normal orientation for transcription and so express an RNA transcript that is complementary to the target mRNA molecule (*i.e.*, the RNA transcript of the anti-sense nucleic acid molecule can hybridize to the target mRNA molecule through Watson-Crick base pairing). An anti-sense nucleic acid molecule may be constructed in a number of different ways provided that it is capable of interfering with the expression of a target protein. Typical anti-sense oligonucleotides to be screened preferably are 30-40 nucleotides in length. The anti-sense nucleic acid molecule generally will be substantially identical (although in antisense orientation) to the target gene. The minimal identity will typically be greater than about 80%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 90% is preferred, though about 95% to absolute identity would be most preferred.

Candidate nucleic acid molecules can possess ribozyme activity. Thus, the methods of the invention can be used to screen for ribozyme molecules that inhibit the functional expression of one or more mRNA molecules that encode one or more proteins that mediate an IL-1 delta dependent cellular response. Ribozymes are catalytic RNA molecules that can cleave nucleic acid molecules having a sequence that is completely or partially homologous to the sequence of the ribozyme. It is possible to design ribozyme transgenes that encode RNA ribozymes that specifically pair with a target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and cleaving other molecules. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the antisense constructs.

The design and use of target RNA-specific ribozymes is described in Haseloff et al. (*Nature*, 334:585, 1988; see also U.S. Patent No.5,646,023), both of which publications are incorporated herein by reference. Tabler et al. (*Gene* 108:175, 1991) have greatly simplified the construction of catalytic RNAs by combining the advantages of the anti-sense RNA and the ribozyme technologies in a single construct. Smaller regions of homology are required for ribozyme catalysis, therefore this can promote the repression of different members of a large gene family if the cleavage sites are conserved.

#### Research reagents

Another use of the polypeptide of the present invention is as a research tool for studying the biological effects that result from IL-1 delta /IL-1 delta counter-structure interactions on different cell types. Alternatively, the polypeptide of the present invention could be used as a research tool for studying the biological effects that result from inhibiting the interaction of the IL-1 delta counterstructure with its agonist ligand. Polypeptides also may be employed in *in vitro* assays for detecting IL-1 delta counter-structure molecules or IL-1 delta polypeptides or the interactions thereof.

IL-1 delta may also be used as a reagent to identify (a) the proteins to which it binds, and which are involved in IL-1 delta signaling, and (b) other proteins with which it might interact which would be involved in signal transduction pathways. These other proteins would then be useful tools to search for other inhibitors of signaling. IL-1 delta could be used by coupling recombinant protein to an affinity matrix, or by using it as a bait in the 2-hybrid system.

The interaction between IL-1 delta polypeptide and its counter-structure enables screening for small molecules that interfere with the IL-1 delta polypeptide/IL-1 delta counter-structure association and therefore inhibit the activity of IL-1 delta polypeptide or its counter-structure. The interaction also enables the screening for molecules that enhance or agonize IL-1 delta activity, as described herein.

To assist in this, the yeast two-hybrid system developed at SUNY (described in U.S. Patent No. 5,283,173 to Fields et al.) may be used to screen for inhibitors of IL-1 delta as follows. IL-1 delta polypeptide and its counter-structure, or portions thereof responsible for their interaction, may be fused to the Gal 4 DNA binding domain and Gal 4 transcriptional activation domain, respectively, and introduced into a strain that depends on Gal 4 activity for growth on plates lacking histidine. Compounds that prevent growth may be screened in order to identify IL-1 inhibitors. Alternatively, the screen may be modified so that IL-1 delta polypeptide/IL-1 delta polypeptide counter-structure interaction inhibits growth, so that inhibition of the interaction allows growth to occur.

Another *in vitro* approach to screening for IL-1 delta inhibition would be to immobilize one of the components (either IL-1 delta polypeptide or its counter-structure) in wells of a microtiter plate, and to couple an easily detected indicator to the other component. An inhibitor of the interaction is identified by the absence of the detectable indicator from the well.

In addition, IL-1 delta polypeptides according to the invention are useful for the structure-based design of an IL-1 delta inhibitor. Such a design would comprise the steps of determining the three-dimensional structure of the IL-1 delta polypeptide, analyzing the three-dimensional structure for the likely binding sites of substrates, synthesizing a molecule that incorporates a predictive reactive site, and determining the inhibiting activity of the molecule.

IL-1 delta DNA, IL-1 delta polypeptides, and antibodies against IL-1 delta polypeptides can be used as reagents in a variety of research protocols. A sample of such research protocols are given in Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 ed. Vol. 1-3, Cold Spring Harbor Laboratory Press, (1989). For example, these reagents can serve as markers for cell specific or tissue specific expression of RNA or proteins. Similarly, these reagents can be used to investigate constitutive and transient expression of IL-1 delta RNA or polypeptides. IL-1 delta DNA can be used to determine the chromosomal location of sequences related to IL-1 delta DNA and to map genes in relation to this chromosomal location. IL-1 delta DNA can also be used to examine genetic heterogeneity and heredity, through the use of techniques such as genetic fingerprinting, as well as to identify risks associated with genetic disorders. IL-1 delta DNA can be further used to identify additional genes related to IL-1 delta DNA and to establish evolutionary trees based on the comparison of sequences. IL-1 delta DNA and polypeptides can be used to select for those genes or proteins that are homologous to IL-1 delta DNA or

polypeptides through positive screening procedures, such as Southern blotting and immunoblotting, and through negative screening procedures, such as subtraction.

### Antibodies

Antibodies that are immunoreactive with the polypeptides of the invention are provided herein. Such antibodies specifically bind to the polypeptides *via* the antigen-binding sites of the antibody (as opposed to non-specific binding). Thus, the polypeptides, fragments, variants, fusion proteins, etc., as set forth above may be employed as immunogens in producing antibodies immunoreactive therewith. More specifically, the polypeptides, fragment, variants, fusion proteins, etc. contain antigenic determinants or epitopes that elicit the formation of antibodies.

These antigenic determinants or epitopes can be either linear or conformational (discontinuous). Linear epitopes are composed of a single section of amino acids of the polypeptide, while conformational or discontinuous epitopes are composed of amino acids sections from different regions of the polypeptide chain that are brought into close proximity upon protein folding (C. A. Janeway, Jr. and P. Travers, *Immuno Biology* 3:9 (Garland Publishing Inc., 2nd ed. 1996)). Because folded proteins have complex surfaces, the number of epitopes available is quite numerous; however, due to the conformation of the protein and steric hinderances, the number of antibodies that actually bind to the epitopes is less than the number of available epitopes (C. A. Janeway, Jr. and P. Travers, *Immuno Biology* 2:14 (Garland Publishing Inc., 2nd ed. 1996)). Epitopes may be identified by any of the methods known in the art.

Thus, one aspect of the present invention relates to the antigenic epitopes of the polypeptides of the invention. Such epitopes are useful for raising antibodies, in particular monoclonal antibodies, as described in detail below. Additionally, epitopes from the polypeptides of the invention can be used as research reagents, in assays, and to purify specific binding antibodies from substances such as polyclonal sera or supernatants from cultured hybridomas. Such epitopes or variants thereof can be produced using techniques well known in the art such as solid-phase synthesis, chemical or enzymatic cleavage of a polypeptide, or using recombinant DNA technology.

As to the antibodies that can be elicited by the epitopes of the polypeptides of the invention, whether the epitopes have been isolated or remain part of the polypeptides, both polyclonal and monoclonal antibodies may be prepared by conventional techniques as described below.

In this aspect of the invention, IL-1 delta and peptides based on the amino acid sequence of IL-1 delta, can be utilized to prepare antibodies that specifically bind to IL-1 delta. The term "antibodies" is meant to include polyclonal antibodies, monoclonal antibodies, fragments thereof, such as F(ab')<sub>2</sub> and Fab fragments, as well as any recombinantly produced binding partners. Antibodies are defined to be specifically binding if they bind IL-1 delta polypeptide with a  $K_d$  of greater than or equal to about  $10^7$  M<sup>-1</sup>. Affinities of binding partners or antibodies can be readily determined using conventional techniques, for example those described by Scatchard et al., *Ann. N.Y. Acad. Sci.*, 51:660 (1949).

Polyclonal antibodies can be readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats, using procedures that are well known in the art. In general, purified IL-1 delta or a peptide based on the amino acid sequence of IL-1 delta polypeptide that

is appropriately conjugated is administered to the host animal typically through parenteral injection. The immunogenicity of IL-1 delta polypeptide can be enhanced through the use of an adjuvant, for example, Freund's complete or incomplete adjuvant. Following booster immunizations, small samples of serum are collected and tested for reactivity to IL-1 delta polypeptide. Examples of various assays useful for such determination include those described in *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures, such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immunosorbent assays (ELISA), dot blot assays, and sandwich assays. See U.S. Patent Nos. 4,376,110 and 4,486,530.

Monoclonal antibodies can be readily prepared using well known procedures. See, for example, the procedures described in U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980. Briefly, the host animals, such as mice, are injected intraperitoneally at least once and preferably at least twice at about 3 week intervals with isolated and purified IL-1 delta or conjugated IL-1 delta peptide, optionally in the presence of adjuvant. Mouse sera are then assayed by conventional dot blot technique or antibody capture (ABC) to determine which animal is best to fuse. Approximately two to three weeks later, the mice are given an intravenous boost of IL-1 delta or conjugated IL-1 delta peptide. Mice are later sacrificed and spleen cells fused with commercially available myeloma cells, such as Ag8.653 (ATCC), following established protocols. Briefly, the myeloma cells are washed several times in media and fused to mouse spleen cells at a ratio of about three spleen cells to one myeloma cell. The fusing agent can be any suitable agent used in the art, for example, polyethylene glycol (PEG). Fusion is plated out into plates containing media that allows for the selective growth of the fused cells. The fused cells can then be allowed to grow for approximately eight days. Supernatants from resultant hybridomas are collected and added to a plate that is first coated with goat anti-mouse Ig. Following washes, a label, such as <sup>125</sup>I-IL-1 delta, is added to each well followed by incubation. Positive wells can be subsequently detected by autoradiography. Positive clones can be grown in bulk culture and supernatants are subsequently purified over a Protein A column (Pharmacia).

The monoclonal antibodies of the invention can be produced using alternative techniques, such as those described by Altling-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid Alternative to Hybridomas", *Strategies in Molecular Biology* 3:1-9 (1990), which is incorporated herein by reference. Similarly, binding partners can be constructed using recombinant DNA techniques to incorporate the variable regions of a gene that encodes a specific binding antibody. Such a technique is described in Larrick et al., *Biotechnology*, 7:394 (1989).

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab and F(ab')<sub>2</sub> fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to



humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen binding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature* 332:323, 1988), Liu et al. (*PNAS* 84:3439, 1987), Larrick et al. (*BioTechnology* 7:934, 1989), and Winter and Harris (*TIPS* 14:139, May, 1993). Procedures to generate antibodies transgenically can be found in GB 2,272,440, US Patent Nos. 5,569,825 and 5,545,806 and related patents claiming priority therefrom, all of which are incorporated by reference herein.

#### Uses Thereof

The antibodies of the invention can be used in assays to detect the presence of the polypeptides or fragments of the invention, either *in vitro* or *in vivo*. Due to the RNA expression pattern of IL-1 delta, it is expected that IL-1 delta polypeptides will be expressed in lymph node, thymus, tonsil, brain, placenta, lung, skeletal muscle, prostate, and testis cells and tissues. Antibodies against IL-1 delta polypeptides can be used to detect lymph node, thymus, tonsil, brain, placenta, lung, skeletal muscle, prostate, and testis tissue and cell types by convention immunohistochemical methods. The antibodies also may be employed in purifying polypeptides or fragments of the invention by immunoaffinity chromatography.

Those antibodies that additionally can block binding of the polypeptides of the invention to IL-1 delta counter-structure molecules may be used to effect a biological activity that results from such binding. The antibodies may either (i) inhibit the activity of IL-1 delta polypeptide, if IL-1 delta is an agonist, or (ii) allow the binding and signaling activity of the IL-1 delta counterstructure ligand, if IL-1 delta is an antagonist. For example, activation of the transcription factors NFkappaB and AP1, the protein kinases Jun N-terminal kinase and p38 map, the enzymes COX-2 leading to prostaglandin production and iNOS leading to nitric oxide production, and inflammation in general may be inhibited. Such blocking antibodies may be identified using any suitable assay procedure, such as by testing antibodies for the ability to inhibit binding of IL-1 delta polypeptides to certain cells expressing IL-1 delta counter-structure molecules. Alternatively, blocking antibodies may be identified in assays for the ability to inhibit a biological effect that results from binding of IL-1 delta counter-structure molecules to target cells. Antibodies may be assayed for the ability to inhibit IL-1 delta counter-structure molecules-mediated lysis of cells, for example.

Such an antibody may be employed in an *in vitro* procedure, or administered *in vivo* to inhibit a biological activity mediated by the entity that generated the antibody. Disorders caused or exacerbated (directly or indirectly) by the interaction of IL-1 delta counter-structure molecules with cell surface (binding partner) receptor thus may be treated. A therapeutic method involves *in vivo* administration of a blocking antibody to a mammal in an amount effective in inhibiting a IL-1 delta counter-structure molecule-mediated biological activity. Monoclonal antibodies are generally preferred for use in such therapeutic methods. In one embodiment, an antigen-binding antibody fragment is employed.

Compositions comprising an antibody that is directed against IL-1 delta polypeptides, and a physiologically acceptable diluent, excipient, or carrier, are provided herein. Suitable components of such compositions are as described above for compositions containing IL-1 delta polypeptides.

Also provided herein are conjugates comprising a detectable (e.g., diagnostic) or therapeutic agent, attached to the antibody. Examples of such agents are presented above. The conjugates find use *in vitro* or *in vivo* procedures.

The references cited herein are incorporated by reference herein in their entirety.

The embodiments within the specification and the following examples provide an illustration of embodiments of the invention and should not be construed to limit the scope of the invention. The skilled artisan recognizes many other embodiments are encompassed by the claimed invention.

#### **EXAMPLE 1**

##### **Recombinant Expression of Human IL-1 delta**

A full length human IL-1 delta expression construct was generated in the following manner. IL-1 DNA encoding IL-1 delta polypeptide was cloned in frame, either without a tag or with a C-terminal FLAG/polyHis tag, between the SalI and NotI sites of the expression vector pDC412. The protein was produced by transfection into COS cells. Human IL-1 delta was secreted with moderate efficiency from transfected COS or CV-1/EBNA cells.

#### **EXAMPLE 2**

##### **Human IL-1 delta RNA Expression**

Expression of human IL-1 delta mRNA was analyzed using 3 Clontech multiple tissue cDNA panels via a nested PCR approach. The three panels used were:

1. Human Panel I (Cat.# K1420-1) Contains heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas first strand cDNAs.
2. Human panel II (Cat.# K1421-1) Contains spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte first strand cDNAs.
3. Human Immune Panel (Cat.# K1426-1) Contains spleen, lymph node, thymus, tonsil, bone marrow, fetal liver, and peripheral blood leukocyte first strand cDNAs.

Analysis was performed by an initial round of PCR followed by a second round of PCR using nested primers.

FIRST ROUND: Primers: 5' sense = BRR464.26 (just upstream of coding region, in 5' UTR)

3' antisense = BRR453.26 (within coding region, near 3' end)

BRR464.26 5' GGGAGTCTACACCTGTGGAGCTCAA 3'

BRR453.26 5' CTGCTGGAAGTAGAAGTCTGTGATGG 3'

## Reaction conditions (per 50ul reaction):

5ul each 1st strand cDNA (or 5ul H2O for negative control or 100ng human genomic DNA), 12.5 pmol each primer -200uM each dNTP, 0.5ul of a 16:1 mixture of KlenTaq1 and Vent polymerases, 50mM Tris-HCl, pH 9.2 -3.5mM MgCl<sub>2</sub>, 16mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 150ug/ml BSA

## Cycling parameters:

1 cycle 98°C, 3min; 58°C, 45sec; 72°C, 45sec  
 28 cycles 98°C, 45sec; 58°C, 45sec; 72°C, 45sec  
 1 cycle 98°C, 45sec; 58°C, 45sec; 72°C, 45sec

SECOND ROUND: Reactions from first round were diluted 1:50 in TE and 1ul of each dilution amplified with nested primers.

Primers: 5' sense = BRR463.30 (spans initiating ATG; downstream of and overlapping first sense primer).  
 3' antisense = BRR462.28 (within coding region, near 3' end; downstream of first antisense primer).

BRR463.30 5' GGAGCTCAAGATGGTCCTGAGTGGGGCGCT 3'

BRR462.28 5' GCATTCCAGCCACCAATTCTCGGAAGCT 3'

Reaction conditions and cycling parameters were identical to that of the first round. 10ul of each reaction from first and second round PCRs were separated by electrophoresis on 1.2% agarose gels. Products were visualized by UV light following staining with ethidium bromide. Expression was detected in lymph node, thymus, tonsil, brain, placenta, lung, skeletal muscle, prostate, and testis.

Expression of IL-1 delta was also analyzed in several animal models of human disease by conventional real-time polymerase chain reaction (RT-PCR) substantially as described in USSN 09/876,790, filed June 6, 2001, and/or by TaqMan® RT-PCR (Applied Biosystems, Foster City, CA). Total RNA from small or large intestine (colitis models: DSS-induced colitis, anti-CD-3 induced ileitis and MdrKO spontaneous colitis), spinal cord (multiple sclerosis [MS] models: EAE using SJL mice injected with PLP), paws (collagen-induced arthritis or CIA, a model of rheumatoid arthritis), or lung (asthma model: BALB/c/OVA-induced asthma model) was used to make first strand cDNA. The level of expression was subjectively scored as a function of relative ethidium bromide staining intensity.

Results of these experiments indicated that IL-1 delta was upregulated in the OVA-induced asthma model, and in the CIA model, indicating that an agent that modulates the activity of IL-1 delta may be useful in treating or ameliorating asthma and other pulmonary conditions relating to an immune or inflammatory response, as well as rheumatoid arthritis and other arthritic conditions that have an inflammatory or autoimmune component.

### **EXAMPLE 3**

#### **Human IL-1 delta Polypeptide purification**

The poly His tag is used to bind the recombinant protein to Nickel-NTA resin (manufactured by Qiagen, <http://www.qiagen.com/>) according to the manufacturer's instructions. The resin is washed with 30

column volumes of 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl + 5 mM Imidazole. The recombinant protein is then eluted using increasing concentrations of Imidazole. Initially a gradient of 5-20mM Imidazole 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl is used, followed by 20mM Imidazole 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl, followed by a gradient of 20-100mM Imidazole 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl, followed by 100mM Imidazole 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl, followed by 500mM Imidazole 20 mM NaPO<sub>4</sub> pH 7.4 + 300 mM NaCl. Fractions are collected and analyzed by SDS-PAGE to identify those containing the recombinant protein.

#### EXAMPLE 4

##### Activation of Signaling Molecules in Human Cells by Human IL-1 delta

The following describes tests and results that are carried out evaluate the induction of some of the same signaling molecules involved in stress responses as are activated by IL-1 alpha, IL-1 beta and other inflammatory cytokines.

Human IL-1 delta is transfected into COS-1 cells. Several days after the transfection, conditioned medium (containing the transiently expressed IL-1 delta) is harvested. Test cells are incubated with this conditioned medium, or alternatively with conditioned medium from COS-1 cells transfected with the empty expression vector. Approximately 10 minutes following the incubation, cell extracts are prepared from the test cells, and the presence of activated signaling molecules is assayed by the use of antibodies specific for the phosphorylated forms of IKBalpha (phosphorylation on Ser32), p38 MAP kinase (phosphorylation on Thr180 and Tyr182), and Stress-Activated Protein Kinase (SAPK/JNK) (phosphorylation on Thr183/Tyr185). The antibodies may be obtained from commercial sources, such as New England Biolabs, Beverly, MA. These signal transduction molecules are known to be involved in a wide range of cellular responses to stimuli such as UV irradiation, endotoxin, and inflammatory cytokines including IL-1 beta. phosphorylation of one or more of these molecules indicates that IL-1 delta is involved in stress response signaling pathways.

#### EXAMPLE 5

##### Activation of Cell Surface Molecules in Human Cells by Human IL-1 delta

The following describes tests that are carried out to evaluate the ability of IL-1 to induce cell surface molecules involved in stress responses (such as those that are induced by IL-1 alpha, IL-1 beta and other inflammatory cytokines).

Human IL-1 delta is transfected into COS-1 cells. Several days after the transfection, conditioned medium (containing the transiently expressed IL-1 delta) is harvested. Human foreskin fibroblast (HFF) cells are incubated for 18 hours at 37degreesC with this conditioned medium diluted 1:1 with fresh 0.5% serum-containing medium, or alternatively with conditioned medium from control COS-1 cells transfected with the empty expression vector, diluted 1:1 with fresh 0.5% serum-containing medium.

Following treatment with the conditioned medium from COS-1 cells, the HFF cells are washed twice with PBS and removed from the tissue culture vessel with versene (non-trypsin reagent). Cell-surface ICAM-1 levels are measured by staining with anti-CD54-PE antibody (Pharmingen, San Diego, CA) on ice

for one hour followed by washing and FACS-based detection. An increase in the level of cell-surface ICAM-1 indicates that IL-1 delta is involved in upregulating cell-surface molecules that are induced during stress response.

#### EXAMPLE 6

##### Modulation of Cytokine Levels by IL-1 delta

The following describes tests that are carried out to evaluate induction of cytokine secretion in dendritic cells or other cells capable of secreting cytokines.

Monocyte-derived dendritic cells (MoDC) are obtained essentially as described by Pickl et al. (J. Immunol. 157:3850, 1996). Briefly, highly purified CD14(bright) peripheral blood monocyctic cells are obtained from peripheral blood using an AutoMACS cell sorting system and anti-CD14 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytic cells are cultured in the presence of IL-4 and GM-CSF for seven days to yield MoDC. Similar techniques are used to obtain purified or enriched populations of other cytokine-secreting cells, for example lymphocytes or granulocytes

Cells are treated for two to three days in the presence or absence of IL-1 delta at varying concentrations; lipopolysaccharide (LPS) at 10ng/ml is used as a positive control; heat-inactivated IL-1 delta (heated at 100 degreesC for 30 minutes) may be used as a negative control. Cells are separated from the supernatant medium by centrifugation.

The supernatant medium is analyzed for soluble cytokine levels using a suitable assay (for example, the Luminex® multi-plex cytokine assay; Luminex Corporation, Austin, TX). Following culture, the supernatant is harvested and assayed for several cytokines including IL-10, IL-2, IL-4, IL-6, IL-8, IL-12 (p70 heterodimer), TNF-alpha, IFN-gamma, and GM-CSF.

For analysis of the induction of cytokine mRNA, the cells are harvested and total RNA is isolated (for example, using an RNeasy® Total RNA System mini-kit, QIAGEN, Venlo, The Netherlands) and analyzed in a suitable, real-time quantitative polymerase chain reaction (PCR) analysis. Quantitative RT-PCR is performed using the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and TaqMan® reagents (Applied Biosystems). An increase in the levels of one or more cytokines and/or induction of one or more cytokine mRNAs indicates that IL-1 delta upregulates cytokines that are involved in the inflammatory and/or immune response.

#### EXAMPLE 7

##### Effect of IL-1 delta on Mixed Lymphocyte Reaction (MLR)

The following describes tests carried out to evaluate the effects of IL-1 delta on TNF-alpha, IFN-gamma, and IL-10 secretion in a mixed leukocyte reaction (MLR) assay.

Briefly, MoDCs are generated as described above. Purified CD3+ allogeneic T cells are obtained from freshly drawn blood using an AutoMACS cell sorting and anti-CD3 magnetic microbeads system (Miltenyi Biotec).

The allogeneic T cells are then mixed with MoDCs at a 1:10 MoDC:T ratio in quadruplicate in the presence of IL-1 delta at varying concentrations from 5 ng/ml to 200 ng/ml, or control preparations. The ensuing mixed lymphocyte reaction (MLR) is allowed to proceed for four days, and supernatants are harvested and assayed for TNF-alpha, IFN-gamma, and IL-10 using a suitable assay as described previously (for example, the Luminex® multi-plex cytokine assay, DELFIA® or ELISA substantially as described below).

### EXAMPLE 8

#### Cytokine ELISA

The following describes an Enzyme-Linked Immunosorbent Assay (ELISA) that is useful to detect and/or quantitate secreted proteins. The Example describes an assay specific for IL-10; those of skill in the art will recognize that a similar assay could be used to detect other molecules.

ELISA plates (for example, Costar® EIA/RIA 96 well easy wash plates, Corning Incorporated Life Sciences, Acton, MA) are coated overnight with 100 microliter of a 2 micrograms/ml mixture of Rat-anti-huIL-10 capture antibody (BD Pharmingen, San Diego, CA) in binding solution (0.1M NaH<sub>2</sub>PO<sub>4</sub>, pH 9.0) at 4degreesC. Plates are washed with wash buffer (phosphate buffered saline, or PBS, 0.5% Tween 20) four times (400 microliters/well/wash), then one time with PBS without Tween. Plates were blocked with 100 microliters of 5% non-fat dry milk in PBS for 1 hour at room temperature (RT), and then washed with wash buffer six times.

Samples and controls are added to separate wells (100 microliters/well); serial dilutions of a standard protein, recombinant HuIL-10 (BD Pharmingen) in PBS + 3%BSA (starting at 10 ng/ml in 3-fold dilutions through 7 points as a standard curve, with an eighth point as a blank) is used to generate a standard curve for quantitation. The plates are incubated for one hour at RT, then washed with wash buffer six times as previously described, and incubated with biotinylated-rat-anti-HuIL-10 (BD Pharmingen; 100 microliters/well of a 200 ng/ml mixture in PBS + 3% BSA) for one hour at RT. The plates are then washed six times with wash buffer as before, and streptavidin-conjugated horse radish peroxidase (SA-HRP; Zymed Laboratories, Inc., South San Francisco, CA; 100 microliters/well of a 1:4000 dilution in PBS + 3% BSA) is added.

After incubating at RT for 30 minutes, the plates are washed for the final time as described above, and color is developed by adding 100 microliters/well of Tetramethylbenzidine (TMB) substrate (a 1:1 mixture of TMB Peroxidase Substrate: Peroxidase Solution, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The plates are incubated for 30 minutes at RT, at which time color development is stopped with 100 microliters/well of 2N H<sub>2</sub>SO<sub>4</sub>. The plates are read at 450nm wavelength on a Molecular Dynamics (Molecular Dynamics, Sunnyvale, CA) plate reader, a standard curve is prepared, and the quantity of IL-10 in the samples determined by comparison to the standard curve.

## EXAMPLE 9

### Cytokine DELFIA

The following describes a DELFIA® (dissociated enhanced lanthanide fluoroimmunoassay; PerkinElmer LifeSciences, Wallac Oy., Turku, Finland) that is useful to detect and/or quantitate secreted proteins. The Example describes an assay specific for IL-10; those of skill in the art will recognize that a similar assay could be used to detect other molecules.

Briefly, DELFIA® plates (i.e., Costar® high binding 96-well plates, Corning Incorporated Life Sciences, Acton, MA) are coated with a detection (or capture) antibody (preferably a monoclonal antibody; 50 microliters of antibody solution containing 2 micrograms antibody/ml in PBS) at 4degreesC for 24 hours. Plates are washed with wash buffer (phosphate buffered saline, or PBS, 0.05% Tween 20) four times (300 microliters/well/wash), then used in an assay or stored.

Fifty microliters each of test supernatants and cell specific controls are added to separate wells of an antibody-coated plate; dilutions of standard proteins are used to generate a standard curve for quantitation. Test supernatants and controls are incubated in the antibody coated plate to allow binding of cytokine to the antibody. Plates are then washed and a polyclonal biotinylated detection antibody is added at a concentration of 10nM in 50 microliters and incubated to allow binding to the captured cytokine. Plates are washed and Streptavidin-Europium (Eu) is added to the plate at a final concentration of 1 nM (0.06 micrograms/ml) in 50microliters and incubated to allow binding to the biotinylated detection antibody. Plates are again washed and 100 microliters of enhancement solution is added to bind the Eu. The Eu in solution is then detected by time resolved fluorescence and the amount of cytokine secreted can be quantitated relative to standards which are added to each plate.

DELFIA® is amenable to full or partial automation (for example, using a Sagian Bioassay core system, Beckman Coulter, Inc., Fullerton, CA, in combination with a plate reader such as a VICTOR2 TM, PerkinElmer LifeSciences), thereby rendering it useful for high-throughput testing.

## EXAMPLE 10

### Preparation of Antibodies to Human IL-1 Delta

Polyclonal antibodies are readily generated from a variety of sources, for example, horses, cows, goats, sheep, dogs, chickens, rabbits, mice, or rats, using procedures that are well-known in the art. In general, purified polypeptides of the invention, or a peptide based on the amino acid sequence of polypeptides of the invention that is appropriately conjugated, is administered to the host animal typically through parenteral injection. The immunogenicity of these polypeptides can be enhanced through the use of an adjuvant, for example, Freund's complete or incomplete adjuvant. Alternatively, DNA encoding IL-1 delta can be used as an immunogen. Following booster immunizations, small samples of serum are collected and tested for reactivity to the polypeptides. Examples of various assays useful for such determination include those described in: *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; as well as procedures such as countercurrent immuno-electrophoresis (CIEP), radioimmunoassay, radio-immunoprecipitation, enzyme-linked immuno-sorbent assays (ELISA), dot blot assays, and sandwich assays, see U.S. Patent Nos. 4,376,110 and 4,486,530.

Monoclonal antibodies are readily prepared using well-known procedures, see for example, the procedures described in U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980. Briefly, host animals, such as BALB/c mice, are injected intraperitoneally at least once, and preferably at least twice at about 3 week intervals with isolated and purified polypeptides or conjugated polypeptides of the invention, optionally in the presence of adjuvant. Preferably, at least about 10 µg of isolated and purified polypeptide of the invention or peptides based on the amino acid sequence of polypeptides of the invention in the presence of RIBI adjuvant (RIBI Corp., Hamilton, Montana) is used. Mouse sera are then assayed by conventional dot blot technique or antibody capture (ABC) to determine which animal produces the highest level of antibody and whose spleen cells are the best candidate for fusion.

Approximately two to three weeks later, the mice are given an intravenous boost of the polypeptides or conjugated polypeptides (such as 3 µg suspended in sterile PBS). Mice are later sacrificed and spleen cells fused with commercially available myeloma cells, such as Ag8.653 (ATCC CRL-1580), following established protocols. Briefly, the myeloma cells are washed several times in media and fused to mouse spleen cells at a ratio of about three spleen cells to one myeloma cell. The fusing agent can be any suitable agent used in the art, for example, polyethylene glycol (PEG) or more preferably, 50% PEG: 10% DMSO (Sigma). The fusion is plated out into, for example, 96-well flat bottom plates (Corning) containing an appropriate medium, such as HAT supplemented DMEM media, and allowed to grow for about eight days. Supernatants from resultant hybridomas are collected and added to, for example, a 96-well plate for 60 minutes that is first coated with goat anti-mouse Ig. Following washes, <sup>125</sup>I-polypeptide or peptides of the invention are added to each well, incubated for 60 minutes at room temperature, and washed four times. Positive wells can be subsequently detected by conventional methods, such as autoradiography at -70degreesC using Kodak X-Omat S film. Other suitable means of identifying antibodies that bind IL-1 delta may be used (including, for example, ELISA, IFA, or one of the aforementioned assays using cells that respond to IL-1 delta).

Positive hybridoma cells can be injected intraperitoneally into syngeneic rodents to produce ascites containing high concentrations (for example, greater than 1 milligram per milliliter) of anti-IL-1 delta polypeptides monoclonal antibodies. Alternatively, positive hybridoma cells can be grown in bulk culture. Monoclonal antibodies are subsequently purified, such as over a Protein A or G column (Pharmacia, Uppsala, Sweden) or by affinity chromatography.

Antibodies can be further tested to evaluate their effects on the ability of IL-1 delta to induce a biological activity (for example, induction of inflammatory cytokines in MoDC or other cytokine secreting cells, induction of ICAM-1 on cells, phosphorylation of IKKα, p38 MAP kinase, and/or Stress-Activated Protein Kinase (SAPK/JNK), or other markers of IL-1 delta biological activity). An antibody that mimics or increases the ability of IL-1 delta to induce a biological activity is referred to as an agonistic antibody, whereas an antibody that decreases the ability of IL-1 delta to induce a biological activity is referred to as an antagonistic antibody. Both types of antibodies may be generated and identified by means that are well known in the art, and will have uses in detection or purification of IL-1 delta as reagents for



research or clinical use, and in therapy and/or diagnosis of conditions mediated by IL-1 delta as described herein.

#### EXAMPLE 11

##### Mouse inflammatory bowel disease models

This example describes several mouse models of inflammatory bowel disease (IBD), which includes Crohn's Disease and ulcerative colitis. Inflammatory bowel disease in animals can either occur spontaneously or can be experimentally induced. It is necessary to exercise care when selecting IBD models to study to ensure that the particular model selected appropriately represents the relevant stage of the inflammatory process under investigation. Particularly useful models of IBD include:

##### A. Oral administration of dextran sulfate sodium (DSS)

The DSS induction model can be used to induce either chronic or acute IBD. In the acute protocol, mice are given DSS (preferably with a molecular weight of 40Kd; from 2% to 8%) in their drinking water for from one to eight days. The percent DSS and the duration of induction will vary depending on the strain of mouse used (for example, C3H/HeJ, C3H/HeJBir, NOD and NOD/SCID mice are highly susceptible, DBA/2, C57BL/6. BALB/c and 129/SvJ mice are moderately susceptible, with varying degrees of susceptibility relative to each other, FVB mice are moderately resistant, and NON/LtJ mice are resistant to DSS induced colitis). In the acute model, DSS is withdrawn after the induction phase. To induce chronic colitis, 2-8% DSS is administered for from 5 to seven days followed by administration of water for ten days; this cycle is repeated three to four times.

DSS-induced colitis is marked by profound inflammation in the colon of animals characterized by crypt destruction, mucosal ulceration, erosions and infiltration of lymphocytes and neutrophils into the mucosal tissue. Histopathologic changes are individually scored as 0 (no findings), 1 (minimal), 2 (mild), 3 (moderate), 4 (severe) for each of the following parameters: increased lymphocytes, increased neutrophils, ulceration, edema, crypt degeneration, and crypt regeneration. Total lesion score, crypt length and number of ulcers are also determined and used to gage severity of colitis.

##### B. Anti-CD3-induced ileitis

Mice (for example, BALB/c, C57BL/6 or MPJ mice, 6-16 weeks of age) are given a single intraperitoneal (i.p.) injection of anti-CD3epsilon antibody or control Ab (50 micrograms diluted in 500 microliters PBS, pH 7.4). In wildtype mice such as those listed above, this treatment reliably induces diarrhea without being lethal. Immunosuppressants such as cyclosporin A (CsA, 50 mg/kg) or dexamethasone (Dex, 50 mg/kg) may be given i.p. either as a single dose at the same time as anti-CD3 antibody, or daily for a total of three injections beginning at the time of anti-CD3 injection, as control molecules that downregulate any ensuing immune response and prevent or ameliorate anti-CD3-induced ileitis.

Mice are monitored for clinical signs of ileitis; mice may be sacrificed at varying time points for histopathologic analysis and/or testing by other means to evaluate apoptosis in gut tissue. For histopathology, hematoxylin and eosin (H&E) stained tissue sections of paraffin embedded intestinal specimens are graded in a blinded fashion, for example by using a quantitative histology score based on the frequency of apoptotic epithelial cells within the epithelium and the ratio of villus height to crypt length. Histological alterations of the small intestinal mucosa that may be observed include a reduced villus height, increased thickness of the crypt region, loss of Paneth cells, goblet cells and IEL in the epithelial layer and severe morphologic changes of the epithelial cells. In the villi, the enterocytes may have lost their columnar and polarized morphology and become flattened. In the crypt region, numerous apoptotic bodies may be identified in the epithelium.

#### C. MdrKO spontaneous colitis

The MDR gene family was identified by an ability to confer multiple drug resistance in cell lines. Three genes have been identified in rodents (*mdr1*, *mdr2* and *mdr3*), and two in humans (MDR1, MDR3). The mouse *mdr1a* gene encodes a 170kDa transmembrane protein that is expressed in many tissues, including intestinal epithelial cells and subsets of lymphoid and hematopoietic cells. Its function in these cells is currently unknown, however, mice deficient in *mdr1a* spontaneously develop colitis. In humans, MDR1 may be associated with IBD susceptibility (Satsangi et al., *Nat. Genet.* 14:199, 1996; Brant et al., *Gastroenterology*, 118:A331, 2000), while decreased MDR1 expression has been reported in mucosal tissue from both CD and UC patients (Lawrance et al., *Hum. Mol. Genet.* 10: 445, 2001; Farrell et al., *Gastroenterology*, 118:279, 2000). *Mdr1a* knockout mice (MdrKO) provide a model of both acute (spontaneous) and chronic (DSS-induced) IBD, similar to that seen in humans, where IBD is generally a mixture of both chronic and acute inflammation. Acute colitis in MdrKO mice is marked by the spontaneous appearance of diarrhea and bloody stools in a subset of the mice; chronic colitis is induced by administering 3% w/v DSS for seven days in drinking water, followed by normal water.

Histopathologic changes are individually scored as 0 (no findings), 1 (minimal), 2 (mild), 3 (moderate), 4 (severe) for each of the following parameters: increased mononuclear cells, increased neutrophils, ulceration, edema, crypt degeneration, and hyperplasia.

#### D. *Helicobacter*-induced colitis

Various strains of mice with immunologic defects (i.e., IL-10<sup>-/-</sup> mice, recombination-activating gene (Rag1)<sup>-/-</sup> mice, T-cell receptor alpha (TCRalpha)<sup>-/-</sup> mice) are susceptible to colitis induced by infection with *Helicobacter* spp., as described in Burich et al. (*Am J Physiol Gastrointest Liver Physiol* 281:G764, 2001). Moreover, luminal bacteria appear to be an important factor contributing to the development of IBD in mice and humans. Accordingly, introduction of *Helicobacter* spp. into immunodeficient mice also serves as an animal model of IBD humans (Burich et al. *supra*). In MdrKO mice, different species of *Helicobacter* may have

different effects on spontaneous colitis; *H. bilis* infection induces IBD at a much earlier age, and the phenotypic appearance of *Helicobacter*-induced disease is similar, but not identical, to spontaneous IBD. In contrast, there is minimal disease in *H. hepaticus*-infected *mdr1a*<sup>-/-</sup> mice, and *H. hepaticus* appears to delay onset of spontaneous IBD. Accordingly, those of skill in the art can utilize a *Helicobacter*-based model of IBD substantially as described by Burich et al. *supra*.

## EXAMPLE 12

### Mouse asthma models

This example describes a mouse model of asthma. Mice (for example, BALB/c) are sensitized with antigen (for example, ovalbumin [OVA]) by intraperitoneal injection of the antigen in alum. Several sensitization schemes are known in the art; a preferred scheme is to inject 10 micrograms of OVA three times at one week intervals (i.e., on day -21, day -14 and day -7). The mice are then challenged with antigen either by aerosol exposure (5 % OVA) or intranasal administration (0.1 mg OVA). The challenge schedule may be selected from among shorter terms (i.e., daily challenge on days 1, 2 and 3) or longer terms (i.e., weekly challenge for two to three weeks). The endpoints that are measured can include airway hyperactivity, bronchoalveolar lavage (BAL) cell number and composition, in vitro draining lung lymph node cytokine levels, serum IgE levels, and histopathologic evaluation of lung tissue. Other animal models of asthma are known, and include the use of other animals (for example, C57BL/6 mice), sensitization schemes (for example, intranasal inoculation, use of other adjuvants or no adjuvants, etc.) and/or antigens (including peptides such as those derived from OVA or other proteinaceous antigens, ragweed extracts or other extracts such as those used in desensitization regimens, etc.).

## EXAMPLE 13

### Mouse collagen induced arthritis model

This example describes two mouse models of rheumatoid arthritis, both of which are induced by immunization with collagen (eg., collagen-induced arthritis or CIA). One model is dependant on tumor necrosis factor (TNF), the other is TNF-independent. Those of skill in the art recognize that other animals models of rheumatoid arthritis exist, and further that various parameters within the models can be adjusted (see, for example, Luross and Williams, *Immunology* 103:407, 2001; Schaller et al., *Nat Immunol* 2:74, 2001; Bober et al., *Arthritis Rheum* 43:2660, 2000, or Weyand, C.M. in *Rheumatology* (Oxford) 2000 June, pgs:3-8)).

TNF-dependent CIA is induced in male, wild-type (wt) DBA/1 mice substantially as a modification of the protocol described by Courtenay, J.S. et al. (*Nature* 283:666, 1980) by immunization of mice with Type II collagen (CII; 100-200 micrograms) in complete Freund's adjuvant (CFA), followed by a booster of CII (200 micrograms) in incomplete Freund's adjuvant (IFA) approximately three weeks later. In untreated mice, CIA manifests in the paws, with increasing severity over time.

TNF-independent CIA is induced in male TNF Receptor double knockout (TNFR DKO) mice substantially as described above. TNFR DKO mice are mice that lack functional TNF receptors (both p55

and p75), and are described in Peschon, et al. (*J. Immunol.* 160:943, 1998). Briefly, mice lacking functional p55 and p75 genes were generated in C57BL/6 background by gene targeting in embryonic stem cells. The TNFR DKO C57BL/6 mice were back-crossed on to the DBA/1 genetic background to yield mice that were homozygous for H-2q and were susceptible to development of CIA.

The severity of disease is judged by swelling and joint function of each paw, using a score from 0 to 4 (0 = normal, no swelling; 1 = swelling in 1 to 3 digits; 2 = mild swelling in ankles, forepaws or more than three digits; 3 = moderate swelling in multiple joints; 4 = severe swelling with loss of function). The score for each paw is totaled for a cumulative score for each mouse; cumulative scores are totaled for the mice in each experimental group to yield a mean clinical score.

#### EXAMPLE 14

##### Mouse experimental allergic encephalomyelitis model

This example describes two mouse models of demyelinating conditions; experimental autoimmune encephalomyelitis (or EAE) is designed to duplicate the secondary, immune mediated demyelination that occurs in multiple sclerosis.

A. Myelin oligodendrocyte glycoprotein (MOG)-induced EAE in C57BL/6 mice

EAE is induced in female C57BL/6 mice substantially as described by Mendel et al. (*Eur. J. Immunol.* 25:1951-59, 1995) by immunization of mice with an antigen derived from rat myelin oligodendrocyte glycoprotein (preferably the MOG35-55 peptide described by Mendel et al., supra). Other encephalitogenic antigens may be used, including, for example, whole spinal cord homogenate, purified whole myelin, myelin basic protein, proteolipid protein, myelin associated glycoprotein, myelin-associated oligodendrocyte basic protein, or encephalitogenic peptides derived from these antigens. The disease induction protocol of Mendel et al. may be modified to include the use of a lower dose of MOG35-55 for immunization (see below), no booster immunization, and the use of RIBI® adjuvant (Corixa Corporation, Seattle WA) instead of complete Freund's adjuvant.

To induce EAE, groups of age and weight-matched mice are given a dose of 100 micrograms of rat MOG35-55 emulsified in 0.2 ml RIBI® adjuvant and injected subcutaneously (for example, at three sites distributed over the shaved flank of a mouse). To induce EAE with accelerated onset, mice may be given an intravenous injection 500 ng pertussis toxin (List Biological Laboratory Inc, Campbell, CA), administered 48 hours after administration of MOG35-55.

B. Proteolipid protein (PLP)-induced EAE in SJL mice

The PLP/SJL model results in a relapsing-remitting course of disease that mimics the course often seen in MS; however, SJL mice are susceptible to anaphylaxis, and care must be given in choosing and administering therapeutic agents to avoid induction of an anaphylactic response. EAE is induced in female SJL mice substantially as described by McRae et al. et al. (*J.*

*Neuroimmunol.* 38:229, 1992) by immunization of mice with an antigen derived from rat proteolipid protein (preferably the PLP13-151(S) peptide described by McRae et al., supra). Other encephalitogenic antigens may be used, including, for example, whole spinal chord homogenate, purified whole myelin, myelin basic protein, proteolipid protein, myelin associated glycoprotein myelin-associated oligodendrocyte basic protein, or encephalitogenic peptides derived from these antigens. The disease induction protocol of McRae et al. may be modified as described above. EAE is reliably induced in SJL/J mice actively immunized with PLP13-151(S) or another, suitable PLP-related antigen. Alternatively, EAE can be induced by adoptive transfer of PLP-specific T cells.

Administration of FIL1 antagonist(s) or control for either or both models is initiated on the day after administration of the encephalitogenic peptide (day 1) and continued through day 11. Varying injection schedules can be used to evaluate the efficacy of the FIL1 antagonist(s). Each mouse is injected intraperitoneally every other day (or according to the selected injection schedule) with 0.2 ml pyrogen-free phosphate-buffered saline (PBS) or 0.2 ml PBS containing FIL1 antagonist(s) or control. Endotoxin levels are monitored and must be less than <10 EU/mg of protein for all reagents. Mice are monitored daily for 30 to 35 days for weight loss, disease onset and severity of clinical signs of EAE by an independent observer blinded to the treatment groups.

The severity of EAE is assessed using either a standard EAE index system in which "0" is used to indicate an asymptomatic mouse and clinical scores ranging from 0.5 to 4 are used to indicate varying degrees of ascending paralysis, or a slightly modified version of the commonly used EAE scoring system. In the latter system, "0" indicates a mouse with no evidence of disease and scores of 1-5 indicate varying degrees of ascending paralysis as follows: 1, tail paralysis; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund or dead. The disease protocol described above induces an acute episode of disease in control mice (peak score of 2-4) from which most recover at least partially. Thus the acute episode of disease is not lethal and mice do not reach a score of 5. The aforescribed scale may be modified to include a score of "0.5" which is given to mice that show the earliest signs of EAE but that do not exhibit complete paralysis of the tail. Mice given a score of 0.5 exhibit some or all of the following symptoms: overnight weight loss of 1-2 grams; noticeable tremor when held up by the tail; and weakness at the distal tip of the tail.

The median day of onset of EAE is determined by Kaplan-Meier Survival analysis. Significant differences in onset between groups are assessed using a Log-Rank comparison. Fischer's exact test is used to analyze the statistical significance of differences in the incidence of EAE among the groups of mice.

#### EXAMPLE 15

##### Mouse cuprizone-induced demyelinating disease model

This example describes a mouse model (cuprizone-induced demyelinating disease or CIDD) that is designed to mimic a type of demyelination that occurs in some cases of multiple sclerosis referred to as primary demyelination. CIDD is induced by feeding cuprizone (bis-cyclohexanone-oxalidihydrazone, a

copper chelator) to mice substantially as described by Matsushima et al. (*Brain Pathol.* 11:107, 2001). At low doses of cuprizone, mature oligodendrocytes in the CNS are specifically insulted and they become unable to provide support for myelin. Demyelination occurs when the damaged myelin is stripped from the axons by microglia.

Some advantages of the CIDD model are that it reproducibly results in massive demyelination in a large area of the mouse brain and it is reversible if cuprizone is removed from the diet. The model appears well suited for profiling gene expression during various stages of demyelination and remyelination. The model has been established in C57BL/6 mice, so it is also suitable for use in KO (knockout) or Tg (transgenic) mice with the B6 background. However, there are no obvious clinical signs associated with the demyelinating process, so analysis must be done by histology.